

Characterization and Modulation of Immune Responses in Mice to a DNA-based Vaccine

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University of Saskatchewan

by

P. Jeffrey Lewis
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DEGREE OF DOCTOR OF PHILOSOPHY

by

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Characterization and Modulation of Immune Responses in Mice to a DNA-based Vaccine

DNA-based vaccines represent a novel method of immunization that has been demonstrated to induce immune responses in animals against a variety of plasmid encoded antigens and following a number of different methods of vaccine delivery.

We characterized the immune response to DNA-based vaccines encoding intracellular, membrane anchored (cell associated) and extracellular (secreted) forms of glycoprotein D (gD), an antigen from the viral envelope of the bovine herpesvirus-1 (BHV-1). Intramuscular injection of mice with plasmids encoding secreted or cell associated forms of this antigen led to seroconversion and a predominance of splenic IFN γ . Mice receiving plasmids encoding cell associated or secreted antigens displayed a predominance of IgG_{2a} and IgG₁, respectively. The predominant serum isotype correlated with the cytokine and antibody isotype profiles within the draining lymph node.

We demonstrated modulation of immune responses in mice following co-delivery of plasmids encoding a secreted form of gD and each of eight different murine cytokines (IL-1 α , IL-12, IL-4, IL-6, IL-10, GM-CSF, IFN γ , TNF α). Plasmids encoding GM-CSF, TNF α , IL-4 and IL-6 demonstrated the capacity to enhance serum IgG titers and seroconversion efficiency. Plasmids encoding IFN γ and TNF α increased levels of serum IgG_{2a} in mice. Varying the dose of plasmids encoding GM-CSF enhanced (10 μ g) or suppressed (50 μ g) serum antibody levels and induced significant increases in IL-4 levels in the spleen and draining lymph nodes. High doses of GM-CSF (50 μ g) increased the levels of serum IgG_{2a} after boosting. Co-administration of plasmids encoding IFN γ either reduced (10 μ g) or enhanced (50 μ g) serum antibody levels and elevated mean serum IgG_{2a} levels.

Finally, we investigated the potential for plasmids encoding the secreted form of gD to elicit immune responses in passively immune mice. We demonstrated that a single intramuscular immunization of passively immune C3H.HeN or C57BL/6 mice with plasmids encoding the secreted form of BHV-1 gD resulted in the development of both cell-mediated and humoral immunity.

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ABSTRACT

DNA-based vaccines represent a novel method of immunization that has been demonstrated to induce immune responses in animals, using a variety of antigens and methods of vaccine delivery. More recently, modulation of immune responses to DNA vaccines has been studied through co-delivery of plasmids encoding a number of different murine cytokines. Most recently scientists have assessed the ability of these novel vaccines to overcome certain problems associated with immunization of passively immune young animals.

We characterized the immune response to several DNA-based vaccines encoding intracellular, extracellular and membrane anchored forms of glycoprotein D (gD), an immunodominant antigen, from the viral envelope of the bovine herpesvirus-1 (BHV-1). BHV-1 is an economically important respiratory pathogen of cattle which is responsible for infectious bovine rhinotracheitis, genital infection, abortion and is also the immunosuppressive primary pathogen of the shipping fever complex. We noted that the kinetics of gD-specific serum antibody development was delayed if the antigen was expressed inside the cell (cytosolic) as compared to expression outside the cell (plasma membrane anchored or secreted). It was also noted that most mice immunized with plasmids encoding secreted, cell surface or cytosolic, versions of gD displayed significant levels of anti-gD antibody, in sera, at 5 and 1/2 months post immunization. Indeed, some mice showed maintenance, or increases, in serum ELISA titers at 5 and 1/2 months post immunization. Intramuscular injection with plasmids encoding secreted or

cell associated forms of BHV-1 gD resulted in a predominance of splenic IFN γ consistent with a Th-1 type of immune response. Surprisingly, despite the involvement of IFN γ in the isotype switch to IgG_{2a}, only mice receiving plasmids encoding the cell associated (plasma membrane anchored and cytosolic) displayed a predominance of this isotype. Mice immunized with plasmids encoding the secreted form of gD displayed a predominance of serum IgG₁. Using cytokine and antibody forming cell (AFC) ELISPOT, we demonstrated that the predominant serum isotype correlated with the cytokine and AFC isotype profile within the draining iliac lymph node.

The humoral and cellular immune responses in C3H/HeN mice following co-delivery of plasmids encoding a secreted form of BHV-1 gD, and plasmids encoding one of eight different murine cytokines (IL-1 α , IL-12, IL-4, IL-6, IL-10, GM-CSF, IFN γ , TNF α), were analyzed. Initially a suboptimal, single dose of plasmid encoding antigen was co-administered with a 10 μ g dose of each of the cytokine expressing plasmids. Data from this experiment demonstrated that co-administration of plasmid encoding GM-CSF enhanced not only early mean serum titers but also the seroconversion efficacy of immunized mice. Plasmids encoding murine TNF α , IL-4 and IL-6 also enhanced the magnitude of serum IgG titers but with differing kinetics. Co-administration of plasmids encoding IFN γ and TNF α increased in the levels of serum IgG_{2a} in some, but not all, immunized mice. In a follow-up experiment where an optimal, fixed, dose of plasmid expressing BHV-1 SgD was co-administered with two different doses of plasmids encoding GM-CSF or IFN γ , we noted that varying the dose of either of these plasmids had a substantial impact on the immunological outcome. Moderate doses of GM-CSF

reproducibly enhanced early mean titers. High doses of GM-CSF resulted in a suppression of humoral responses, however, boosting with high doses of this cytokine resulted in an extremely potent memory response that resulted in very high serum titers and a surprising increase in the levels of serum IgG_{2a}. Assessment of cytokine secretion, within draining lymph nodes and the spleen, showed that co-administration of plasmids encoding GM-CSF induced significant increases in IL-4 secretion after boosting. Co-administration of plasmids encoding IFN γ resulted in the reduction of antibody responses, at moderate doses, that persisted regardless of boosting. Conversely, mice immunized with high doses of this cytokine showed enhanced mean serum antibody titers and benefited from boosting. Mice immunized with both moderate and high doses of plasmids encoding IFN γ displayed elevations in mean serum IgG_{2a}.

Finally, we chose to investigate the potential for plasmids encoding the secreted form of gD to elicit immune responses in passively immune mice following intramuscular immunization. In these experiments we passively immunized 6-7 week old female C3H/HeN or C57BL/6 mice with hyperimmune antisera raised against BHV-1 recombinant, truncated (secreted), gD immediately prior to immunization with plasmid encoded antigen. These studies demonstrated that a single immunization of passively immune mice with plasmids encoding the secreted form of BHV-1 gD resulted in the development of both cell mediated immunity and antibody responses. We also showed that co-administration of plasmids encoding GM-CSF at 25 μ g/ mouse had a detrimental effect on antibody levels and seroconversion efficacy in passively immune mice.

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ABBREVIATIONS USED IN THIS WORK

a.a.	amino acid
Ab	antibody
AgD	authentic (membrane anchored) BHV-1 gD
BCIP	5-bromo-4-3-indolyl phosphate
beta -Gal	beta -galactosidase
BHV-1	bovine herpesvirus-1
BRSV	Bovine respiratory syncytial virus
BRV	Bovine rotavirus
CgD	Cytosolic version of gD
CLDC	cationic liposomes:DNA complexes
CMI	cell mediated immunity
CRPV	Cottontail rabbit papillomavirus
CTL	Cytotoxic T Lymphocyte
DHBV	Duck hepatitis B virus
DMEM	Dulbecco's minimal essential media
DNA	Deoxyribonucleic acid
DOPE	dioleoylphosphatidyl-ethanolamine
DOTAP	dioctadecenoyltrimethylammonio propane-diol
DOTIM	1-{2-(9(Z)-Octadecenoyloxy)-ethyl}-2-(8(Z)-heptadecenyl)-3-(2-hydroxyethyl)-imidazolinium chloride

DTH	delayed type hypersensitivity
E.coli	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot
<i>env</i>	gene encodes two envelope glycoproteins
FBS	fetal bovine serum
FIV	Feline immunodeficiency virus
<i>gag</i>	gene encoding matrix, capsid and nucleic acid-binding proteins
GAP-DLRIE	(±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(docecyl-oxy)-1-propanaminium bromide
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
GFP	green fluorescent protein
GM-CSF	granulocyte/monocyte-colony stimulating factor
H1	hemagglutinin subtype 1
HA	hemagglutinin
HBSS	Hanks buffered salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus

HCMV	Human cytomegalovirus
HEPES	(N-{2-Hydroxyethyl}piperazine-N-{2-ethanesulfonic acid})
HIV-1	Human immunodeficiency virus -1
HSV-1	Herpes simplex virus-1
I.B.R.	infectious bovine rhinotracheitis
i.d.	intradermal
IHNV	Infectious hematopoietic necrosis virus
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal
I.P.V.	infectious pustular vulvovaginitis
ISS	immunostimulatory sequences
i.t.	intratracheal
IFN α , β , γ	interferon alpha, beta, gamma
IgG	immunoglobulin G
IL-#	interleukin
kb	kilobases
kDa	kilodalton
KLH	keyhole limpet hemocyanin
MAb	monoclonal antibody
MCMV	Murine cytomegalovirus
MDBK	Madine Darby Bovine Kidney

MEM	Eagle's minimal essential media
MHC I/II	major histocompatibility complex I and II
NBT	nitroblue tetrazolium
n.d.	not done
<i>nef</i>	gene encodes a protein involved in viral replication
NP	nucleoprotein
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl flouride
<i>pol</i>	gene encoding a reverse transcriptase and integrase
PRV	Pseudorabies virus
<i>rev</i>	gene encoding protein involved in viral replication
RIPA	radioimmunoprecipitation assay
RSV	Rous sarcoma virus
RSV-LTR	Rous sarcoma virus-Long terminal repeat
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SgD	secreted form of gD (BHV-1)
sHA	secreted hemagglutinin
s.n.	serum neutralization
s.q.	subcutaneous
SV-40	simian virus-40

TCR	T-cell receptor
TNF α	Tumor necrosis factor alpha
Th	Helper T-cell
<i>vif</i>	gene encodes protein involved in generation of infectious viral particles a.a.

1.0 LITERATURE REVIEW

1.1 DNA-based vaccines

Immunization of animals with naked nucleic acid encoding antigens under the control of a variety of gene regulatory elements has been described as: polynucleotide immunization, DNA-based vaccination, somatic transgene vaccination, genetic vaccination and nucleic-acid vaccination (Gerloni et al., 1997; Robinson, 1997). Regardless of the terminology, and despite the need for technical correctness, most researchers have settled on DNA-based, or simply DNA vaccines to describe this novel method of immunization. DNA-based immunization was first described in 1992 despite the fact that the evidence for delivery, uptake and expression had been described much earlier (Chattergoon et al., 1997). Since that time detractors have described DNA-based vaccination as "Biological cold fusion", while proponents have termed this unique form of immunization as the "Third Revolution in Vaccinology" (Waine and McManus, 1995). DNA-based vaccination is, without question, well beyond the less than flattering comparison to "Cold Fusion". To date, well over four hundred publications have appeared in respected scientific journals since the first publication coined the phrase "Genetic immunization", in 1992 (Tang et al., 1992). The inherent simplicity of the technology is

unquestionably revolutionary and it is this simplicity that has, in part, driven detractors to distraction. Certainly the simple eloquence of DNA vaccine technology is in stark contrast to the inherently complex forms of vaccine construction such as attenuated and attenuated recombinant pathogens, recombinant subunit vaccines and adjuvants research. Despite the technical simplicity of DNA-based vaccines, it was over 30 years from the time researchers first demonstrated immune responses following injection of naked DNA to the first published description of "genetic immunization" (Atanasiu, 1962; Tang et al., 1992). That simple leap of logic from *in vivo* introduction of naked genetic material, to immunogen synthesis and immune response was as surprising as it was profound.

1.1.1 Injection Grade Plasmid Preparation

From a purely technical standpoint, systemic immunization of animals with naked nucleic acid is eloquent in its simplicity. Plasmid DNA containing any of a variety of eukaryotic gene regulatory sequences, in the appropriate context of a gene, or genes, encoding antigen, are injected intramuscularly (i.m.) or intradermally (i.d.). Purification of amplified plasmid is typically carried out using cation exchange affinity columns with or without removal of endotoxin (Davis et al., 1996). Traditional techniques such as cesium chloride (CsCl) centrifugation may also be used, however, injection of quality DNA typically requires a double CsCl centrifugation usually followed by dialysis. This centrifugation and dialysis procedure is time consuming, hazardous and results in substantial plasmid loss. Despite the limitations of CsCl purification, some researchers have chosen to move away from column purification protocols and back to density

gradient purification claiming enhancement of immunization effectiveness and experimental reproducibility (David Emery, personal communication). At the present time issues regarding plasmid preparation protocols and correlation to immunization efficacy remain unresolved and may simply reflect differences in such things as plasmid sequences, bacterial host cell types and/or growth media. Undoubtedly, as interest in immunostimulatory sequences (ISS's) encoded within bacterially derived DNA, as well as, inclusion of immune modulatory genes grows, researchers are moving towards a greater degree of standardization of plasmid purification protocols. This move will ensure that data reproduction and accurate interlaboratory data comparisons are made possible and reliable.

1.1.2 Delivery of DNA Vaccines

Regardless of purification protocol differences, injection-grade supercoiled plasmid DNA may be stored for long periods of time or resuspended immediately in sterile saline or phosphate buffered saline (PBS). Intramuscular or intradermal delivery may be carried out using standard 0.3-0.5 mL sterile syringes and small gauge hypodermic needles to inject the vaccines. There are several other methods described for delivery of plasmid based vaccines that include: ballistic delivery (Tang et al., 1992; Pertmer et al., 1995), cationic liposomal or microsphere encapsulation (Huang and Wang, 1989; O'Hagan et al., 1993; Keller et al., 1996; Gregoriadis et al., 1997; Jones et al., 1997), and use of bacterial or host cell carriers (Gerloni et al., 1997; Manickan et al., 1997; Sizemore et al., 1997). Ballistic delivery of DNA-based vaccines involves co-

precipitation of plasmid DNA with gold or tungsten microparticles (0.95 - 2.6 μm) such that DNA adsorbs to the surface of the beads. Beads are then "fired" into epidermal surfaces resulting in *in vivo* transfection of a variety of dermal cell types including keratinocytes, fibroblasts and Langerhans cells (Raz et al., 1994). Aerosol or droplet instillation of naked DNA on mucosal surfaces, in the absence of any facilitators, has also elicited immune responses. Mucosal and transdermal (Li and Hoffman, 1995) delivery of DNA vaccines may be facilitated by incorporating DNA-based vaccines into a variety of cationic liposomal preparations or biodegradable microspheres (Alpar et al., 1997; Barnfield et al., 1997). Finally, attenuated bacterial vectors, such as *Shigella flexneri*, which normally infect via intestinal mucosa and also penetrate and persist within host cells, have been specifically mutated such that lysis occurs within host cells. This intracellular lysis releases any plasmid DNA carried by these bacteria into the cytoplasm of the host cell which then sets the stage for subsequent translocation to the nucleus of cells. A number of intracellular pathogens including *Listeria* and *Salmonella* sp. infect via mucosal routes and penetrate host cells. These bacteria provide a mechanism to immunize against the delivery vehicle, as well as, a potentially efficient method of immunizing against a variety of antigens encoded within plasmid DNAs used to transform these bacteria. All of these methods have been demonstrated to deliver intact plasmid DNA to sites of interest with expression of encoded reporter genes or immunogens. Ultimately the purpose of these facilitated delivery systems is to carry, protect and enhance cellular uptake of encapsulated DNA sequences.

1.2 Serum Kinetics and Uptake Mechanisms

From the few pharmacokinetic studies done we know that intravenous (i.v.) injected plasmid DNA (naked or packaged in liposomes) has a serum (extracellular) half-life of <5-10 minutes and that the majority of injected DNA is degraded by serum nucleases and cleared from the body through the kidneys (Kawabata et al., 1995). It is also known that i.v. injected DNA tends to distribute to selected tissues such as the liver, spleen, and skeletal muscle and that muscle associated intracellular DNA appears to persist the longest *in vivo* (Lew et al., 1995). We have demonstrated that i.m. injected DNA travels from the injection site to the draining lymph nodes within minutes of injection (see section 3.3.5). It is not known what percentage of, or indeed if any, DNA that enters the draining lymph node actually passes out of the node and spreads systemically. Despite the polymerase chain reaction (PCR) evidence, showing efflux of DNA from injection sites we do not, at the moment, know if this DNA is cell associated (intracellular or cell surface) or free in the serum. However, we can safely predict that the vast majority of injected DNA is rapidly degraded and likely cleared through the kidneys.

When Wolff et al. (1992) first demonstrated *in vivo* transient transfection of myocytes it was proposed that these cells were unique in their ability to take up plasmid DNA. This was felt to occur as a result of the substantial network of T-tubules that penetrated deeply into myocytes. It was also postulated, based primarily on electron microscopic studies, that uptake may be facilitated by non clathrin-coated pits called caveolae (Anderson et al., 1992; Wolff et al., 1992; Danko and Wolff, 1994). However, in both instances no discussion was presented as to how a relatively large, polyanionic

and hydrophilic molecule, could successfully penetrate the charged lipid bilayer into the largely anionic cell cytoplasm. Further explanations for this mystery were not immediately forthcoming and, indeed, with the demonstration that nonmyocytic cells such as keratinocytes, fibroblasts, and epithelial Langerhans cells were also able to take up naked plasmid DNA the answer seemed an even greater mystery than earlier (Raz et al., 1994; Condon et al., 1996; Casares et al., 1997). Indeed there has been little research, in the context of DNA-based vaccines, to understand how plasmid DNA is perceived at the cell surface let alone translocated into the cytoplasmic compartment and then to the nucleus. In the absence of direct data we are left to rely on several unrelated studies that allow us to theorize on several additional possible mechanisms of plasmid DNA uptake.

Naked DNA uptake by cells, *in vivo*, may be occurring by either a nonspecific method akin to a phagocytic or pinocytotic process, or by a specific receptor-mediated mechanism. Certainly phagocytic uptake of DNA is possible and it has been demonstrated that cytoplasmic access can occur through "leaky" phagosomal membranes within the cytoplasm (Kovacs-ovics-Bankowski and Rock, 1995). Receptor mediated uptake is also plausible although the specific mechanism by which specifically bound plasmid DNA is translocated into the cytoplasm is still unknown (Bennett et al., 1985). DNA-binding cell surface receptors include: the macrophage scavenger receptors, which are capable of binding a variety of molecules including polyribonucleotides, such as, poly(I) and poly(G), and a 30 kd surface receptor, first identified on cancerous cells, found to bind 4500 bp fragments of genomic DNA (Krieger et al., 1985; Bennet et al., 1988; Kimura et al., 1994). This receptor appears to be very specific and also appears to

be restricted to such populations as antigen presenting cells, B-cells and T-cells. There is also preliminary evidence that polyguanylate sequences may facilitate receptor mediated uptake. This receptor complex has been shown to be internalized from the cell surface and there is evidence that some of this internalized DNA is degraded.

Packaging and protection of plasmid based vaccines within cationic liposomes, cochleates, dendrimers or ballistic delivery otherwise may seem to make further research into mechanisms of naked DNA redundant, however, a clear understanding of the modality of uptake and translocation may result in the reduction of the need for cytofectins and ultimately enhance the cost effectiveness of this novel form of immunization. A parameter that is uniquely important in the development of efficacious and cost effective vaccines for the food animals industry.

1.3 Mechanism of Antigen Presentation

Despite the fact that i.m. injection has been the most prevalent route of delivery for DNA-based vaccines the details regarding mechanism of immunity are, at least hypothetically, more clearly understood with dermal and intradermal delivery of DNA. Initially researchers were lead to believe that i.m. delivery was the most efficacious mode of delivery due the presence of unique T-tubular network which presumably allowed penetration of the injected DNA deep within the cells and conceivably facilitated cytoplasmic access of injected DNA. Certainly i.m. injected, plasmid encoded, reporter genes such as β -galactosidase clearly demonstrated that myocytes were effectively transfected and, depending on the protocol and construct, could be transfected, at least

transiently, with efficiencies approaching 30% (Hartikka et. al., 1997). Despite the demonstration that MHC molecules could be upregulated on myoblasts, and to a lesser extent myocytes, and that these cells were capable of producing the cytokine interleukin (IL)-15, it was shown that these cells were incapable of synthesis of important costimulatory molecules such as B7 or CD40 ligand. This information, in conjunction with the inability of myocytes to traffic to draining lymph nodes led researchers to predict that transfected antigen producing myocytes were highly unlikely candidates to be directly involved in priming and expansion of antigen-specific T and B-cell receptor repertoire. Indeed it has been demonstrated recently by several authors that myocytes transfected with plasmids "hand-off" antigen to professional antigen presenting cells such as dendritic cells or macrophages. An important experiment confirmed suspicions that myocytes transfected *in vivo* were indirectly involved in T-cell priming and that direct *in vivo* transfection of professional antigen presenting cells was not an absolute requirement for priming responses (Ulmer et al., 1996c). Ulmer et al. (1996) injected myoblasts of H-2^k haplotype, transfected *in vitro* with plasmid encoded Influenza virus nucleoprotein antigen, into F1 hybrid mice derived from a parental cross of H-2^k and H-2^d haplotypes, and found CD8⁺ cytotoxic T-cell (CTL) responses developed to both haplotypes. Similar studies were carried out by others confirmed the theory that the transfected myocyte was primarily a source of antigen that could be accessed through the normal release of secreted protein or release of cell associated antigen through, antigen "leakiness", overproduction related cytotoxicity, or through antigen-specific CTL activity (Corr et al., 1996; Iwasaki et al., 1997). While this theory is still plausible and may explain the

longevity of humoral and cell mediated immune (CMI) responses associated with DNA-based vaccination it has come under fire recently (Feltquate and Robinson, 1997).

We have demonstrated, using PCR, that within minutes after an i.m. injection of plasmid encoded antigen the gene sequence could be detected within the draining lymph node (see section 3.3.5). However, no one, to date, has demonstrated the presence of plasmid DNA within draining lymph nodes 20 hours or more after i.m. injection, which suggests that intracellular localization within this compartment is minimal. Also, "Van Gogh" experiments, in which tissues, including muscles, receiving plasmids have been amputated at various time points following injection suggest that excision of DNA immunized muscle within 1 minute of DNA delivery, had no effect on the initial immune response (Torres et al., 1997). This work suggests that transfection of myocytes is irrelevant to the developing immune response and that critical transfection of cells distal (lymphatics and draining lymph node) to the injection site or possibly the extremely rapid exit of transfected resident interstitial dendritic cells must occur. The latter explanation seems unlikely in that it assumes that unfacilitated transfection (or at least a stable receptor mediated pre-uptake association with cells) is an extremely rapid occurrence and that maturational and/or mobilization signalling must occur almost simultaneously with DNA-binding. There remains controversy associated with these studies. For example, some studies have demonstrated that excision of skin sites within minutes of ballistic delivery of plasmid DNA had little impact on the developing immune response (Taubes, 1997). These initial observations contradict more recent information and leaves considerable ambiguity with regard to plausible explanations for

the mechanisms of immune response to naked DNA delivered i.m. or i.d. (Torres et al., 1997). Thus, from a mechanistic standpoint the contribution of transfected myocytes, or any nonprofessional antigen presenting cell at the injection site, to the immune character is very much in question. While it was not surprising that myocytes do not play a direct role in priming naive T and B-cells it will be surprising if they are found to play no role whatsoever in some aspect of the immune response. Perhaps longer term studies will demonstrate that myocytes, by virtue of a relatively long period of plasmid encoded gene expression (≥ 30 -60 days) or "bolus" antigen release following antigen-specific CTL activity do contribute to the durability of the immune response by providing a reservoir of antigen that serves to sustain B and T-cell responses (Bachman et al., 1994; Bachman et al., 1996; Neuberger, 1997; Robinson and Torres, 1997).

The demonstration that a multiplicity of cell types that include: keratinocytes, fibroblasts and cells with Langerhans cell morphology, were able to take up, in the absence of any cytofectins, i.d. injected plasmid DNA and express an encoded reporter gene was perhaps, pivotal, in allowing a greater understanding of the mechanistic possibilities of immune responses to DNA-based vaccines (Fynan et al., 1993; Raz et al., 1994). This work, in the context of maturation and mobilization studies on peripheral dendritic cells, allowed the prediction that injection site transfection of Langerhans cells and subsequent maturation and migration to draining lymph nodes, was a likely mechanism for priming of T and B-cell responses (Austyn, 1996; Lutz et al., 1996). Several recent articles have established that *in vitro* and *in vivo* transfected Langerhans cells are able to localize within the draining lymph node and are of critical importance in

the initiation and expansion of the developing immune response (Condon et al., 1996; Manickan et al., 1997). Thus research into dermal/epidermal delivery allowed investigators pursuing i.m. delivery of DNA vaccines to develop a more accurate picture of the underlying mechanism of immunity following immunization with plasmid DNA.

Apart from the issue of the role the transfected myocytes contribute to the overall immune character, there remains a significant question that revolves around the possibility that a lymph node resident population of relevant professional antigen presenting cells, or for that matter antigen producing cells such as fibroblasts, endothelial cells or stromal cells, are transfected by the "wave" of antigen encoding DNA that washes into the node following injection. If this occurs then one must ask what, if any, role these node resident cells play in the overall immune response. Certainly the literature suggests that many interdigitating, and possibly follicular dendritic cells, within the lymph node represent a mature population that have arrived via afferent lymphatics (murine models)(Steinman et al., 1997). Presumably, mature antigen presenting cells within the paracortical regions would, in the absence of an efficient endogenous to exogenous antigen processing pathway, present plasmid encoded antigen primarily in the context of MHC I. If this is the case then distally transfected professional antigen presenting cells would preferentially expand the CD8⁺ CTL population. Certainly expansion of CTL populations is an important feature of DNA-based vaccines. However, one might predict that dendritic cells transfected at the site of injection, expressing cell surface or secreted antigen, would, upon migration to the draining lymph node, drive the priming and expansion of CD4⁺ T helper population and ultimately B-

cell responses. The successful and efficient priming of naive lymphocytes would require presentation of antigen derived epitopes in the context of class II MHC and, depending on the rate of plasma membrane turnover (cell surface antigen) or maintenance of antigen uptake and processing (secreted antigen) functions.

In an attempt to acquire a clearer understanding of the cells involved in induction of the immune response following immunization with naked DNA encoding antigen, it is important to separate the immune response into priming and anamnestic responses. In this context it is a relatively simple task to predict that phagocytically active epidermal Langerhans cells, acquiring antigen either through direct transfection from introduced DNA, or indirectly through phagocytosis of antigen produced locally by transfected keratinocytes or fibroblasts, are key components initiating the immune responses in naive animals. This model predicts a requirement for access to the exogenous pathway of antigen processing and presentation of antigen derived T-cell epitopes via class II MHC molecules either through highly efficient phagocytic mechanisms or, following direct transfection, an endogenous to exogenous antigen processing crossover. Support for this mechanistic prediction is substantial, indeed recent studies utilizing Langerhans cells transfected *in vitro* and *in vivo* with plasmid expressing the marker gene for Green Fluorescent Protein (GFP) have clearly shown trafficking of transfected Langerhans cells to the paracortical regions of the draining lymph node (Condon et al., 1996). Of course the priming T-cell response must have the initial involvement of an immature population of dendritic cells that possess the capacity to take up and process large amounts of material, particularly if the gene(s) within the plasmid encode secreted

antigen. Alternatively interdigitating dendritic cells, although hypothetically exhibiting greatly diminished phagocytic capacity, upon transfection with plasmid encoding membrane anchored antigens may result in significant MHC II loading and subsequent CD4⁺ T-cell priming as a result of normal cellular "housekeeping" and membrane turnover (Guery and Adorini, 1995). Furthermore predicted primary naive B-cell interaction with antigen may occur within the paracortical region if membrane anchored antigen is being produced by transfected interdigitating dendritic cells. However, the possibility for B-cell receptor engagement of soluble antigen (secreted or intracellular) may occur prior to entry into the lymph node and is not necessarily restricted to the paracortical zone. While histological and immunocytochemical information abounds with regard to skin immunology and resident cell populations, this information is less evident in myocytic tissues such as skeletal muscle. Although an interstitial population of dendritic cells has been identified within cardiac muscle it is unknown if a similar population occurs within skeletal muscle. Despite this paucity of information, predictions can be made regarding the mechanism of immune priming based on what is known to occur following i.m. introduction of plasmid encoded genes. First, myocytes are transfected relatively efficaciously following i.m. injection (Wolff et al., 1990; Wolff et al., 1991; Hartikka et al., 1996). Muscles injected with plasmid encoding the reporter gene β -galactosidase clearly show considerable ability to express this protein to a very high degree following uptake and nuclear localization of plasmid. Second, myocytes are, immunologically, incapable of priming T-cell responses directly and must "hand-off" antigen to professional antigen presenting cells in order to initiate the immune response

(Hohlfeld and Engel, 1994; Ulmer et al., 1996c; Torres et al., 1997). Third, this priming population of professional antigen presenting cells are likely dendritic-like cells because studies utilizing *in vitro* transfected antigen presenting cells subsequently introduced into naive animals described immunity only if the transfected cells were dendritic cells and not macrophages (Manickan et al., 1997). More recently, it was demonstrated that *in vivo* transfection of splenic B-cells with plasmid encoding a heavy chain immunoglobulin displaying a B-cell epitope within the complementarity determining region 3 resulted in the development of a significant humoral response (Gerloni et al., 1997). These researchers limited expression to B-cells by utilizing immunoglobulin promoter enhancer elements and demonstrated that transgene integration into the B-cell genome occurred with a high frequency (Xiong et al., 1997). Thus it would appear that transfected B-cells are capable of contributing to the ensuing immune response, although it is unknown at this time why coligation of B-cell receptors and Fc γ RIIB1 do not inhibit the humoral response (D'Ambrosio et al., 1995). Fourth, a wave of injected plasmid DNA washes into the draining lymph node within minutes of injection supporting the possibility that extramuscular transfection may occur. Finally, recent studies involving "Van Gogh" experiments, in which the injected muscle bundle was excised within minutes of injection, had little impact on immunological priming or the early anamnestic response. This final piece of evidence is very compelling and suggests that a population of skeletal muscle resident dendritic cells are, even if they exist, of questionable relevance to the developing immune response. Furthermore it also suggests that myocytic injection site recruitment of immature dendritic cells would be of questionable use, even if it did occur,

to the development of the priming immune response. Therefore we must conclude that the relatively high doses of i.m. injected DNA required to induce immunity are needed to effectively transfect critical immune surveillance cells distal to the site of injection (within draining secondary lymphoid tissues).

As stated previously, a variety of cell types can be transfected *in vivo* in the absence of any uptake-facilitating cytofectins. However, given that antigen presenting cell acquisition of antigen occurs indirectly (Ulmer et al., 1996c) and that normal resident and recent immigrant dendritic cells, in the course of normal maturation, undergo significant phagocytic downregulation one might predict that transfection of virtually any cell type (including afferent lymphatic endothelial cells, subcapsular lining macrophages or fibroblastic reticular cells) would require the presence of an immature, phagocytically active population of dendritic cells. While it has been demonstrated that B-cells and macrophages have the capacity to prime naive T-cells, these antigen presenting cells are generally less efficient than dendritic cells in this regard (Ziegler and Unanue, 1981; Romani et al., 1989; Steinman, 1991; Cassell and Schwartz, 1994; Guery et al., 1996). This may, in part, be a result of the normal architectural constraints within the lymph node that are designed to bring together T-cells (and possibly naive B-cells) in the context of interstitial dendritic cells within the paracortical corridors (Steinman et al., 1997). The larger doses typically utilized with DNA-based vaccines injected i.m. or i.d. may reflect the diminished efficacy of macrophage or B-cell priming of T-cells and the need to transfect antigen presenting cells, including dendritic cells, at some threshold level of efficacy. In light of recent data describing the potent immunostimulating, or adjuvant,

capacity of bacterially derived DNA (Pisetsky, 1996a) it would not seem unreasonable to hypothesize that recruitment of immature dendritic cells to the distal afferent lymphatic, subcapsular sinus or cortical regions of the draining lymph and subsequent uptake and processing of antigen expressed by resident nonprofessional antigen presenting cells. For the moment it appears likely that transfection of cell populations distal to the i.m. injection site occurs, however, the exact role these populations play in development and maintenance of immune responses remains unclear.

1.4 Adjuvancy Effects of Bacterially Derived DNA

Recently, bacterially derived DNA (genomic and extrachromosomal) has been described as a member of bacterial, innate immune, activating substances that include: lipopolysaccharide (LPS), teichoic acid or peptidoglycans (Pardoll and Beckerleg, 1995; Krieg, 1996; Pisetsky, 1996a; Pisetsky, 1996b; Klinman et al., 1997; Sparwasser et al., 1997). The intrinsic adjuvant capacity of bacteria had been described as early as 1984 (Tokunaga et al., 1984), however, it is only within the last four to five years, coincidental with the first publications describing immune responses to DNA-based vaccines, that details of nucleic acid adjuvant effect have emerged (Sato et al., 1996; Sun et al., 1997). At present three distinct elements of immunostimulatory sequences (ISSs) have been determined and/or postulated to play pivotal roles in the activation of the innate immune response, as well as, deviation of immunity towards a Th1 type phenotype. First, eukaryotes and plants exhibit what is termed "CpG suppression" and is characterized by a 20 fold lower than expected occurrence of the CpG dinucleotide pairs within genome

nucleotide sequences. Conversely, bacterial, certain viral and some nonvertebrate DNA sequences have been demonstrated to exhibit the expected number of CpG dinucleotide pairs within the coding and noncoding regions (Yamamoto et al., 1992). Second, the CpG motifs that do occur in eukaryotic DNA are all methylated at the cytosine nucleotide whereas the equivalent motif within bacterial chromosomal or extrachromosomal DNA is hypomethylated (Bird, 1987; Hergersberg, 1991; Pisetsky, 1996a). Finally, 5' and 3' DNA sequences immediately adjacent to the CpG motifs can determine both, efficacy of the target cell association of the DNA fragment and stimulatory capacity of the core sequence. Currently it is believed that a PuPu-CpG-PyPy hexamer is the most efficacious stimulatory hexamer, although this data was originally obtained by assessing blastogenesis and IgM production in DBA/2 and C3H/HeJ mice (Krieg et al., 1995; Sato et al., 1996). It has also been demonstrated that short synthetic 5'-NPu-CpG-PuN-3' oligonucleotides with no additional flanking sequences are also stimulatory but only when prepackaged in cationic liposomes prior to delivery to murine splenocytes (Sonehara et al., 1996). It has also been demonstrated that flanking sequences outside of this hexamer core must include a guanine rich region to ensure binding, and subsequently uptake, by target cells (Kimura et al., 1994). These observations suggest that the hexameric ISSs must penetrate the cytosol of target cells to elicit responses. The exact mechanism of DNA association and uptake and cytoplasmic delivery is unknown, however, there is evidence that the potential quadruplex polyanionic structure, that arises due to the polyguanylate sequence domains, binds with high affinity to the polycationic regions of the collagen-like domain of macrophage scavenger receptor I (Kimura et al.,

1994). It has been suggested that upon entry into the cytosol, CpG motifs bind to the cAMP responsive element binding protein (Sonehara et al., 1996). Indeed, addition of the cAMP agonist forskolin results in the inhibition of ISS induced upregulation of IFN γ . The responses of professional antigen presenting cells, T-cells and NK cells following stimulation with hexameric ISS's are wide ranging and profound and includes: blastogenesis, IgM secretion and upregulation of cytokine expression and secretion.

Cytokines induced by ISSs include: TNF α , IFN α , and β , IFN γ , IL-6, IL-12 and IL-18 (Halpern et al., 1996; Klinman et al., 1996; Yi et al., 1996b; Roman et al., 1997; Sparwasser et al., 1997). All but one of these cytokines have been shown to be derived from ISSs stimulated macrophages, while B-cells appear to be the major source of IL-6 and some IL-12. Inbred mouse strains including BALB/c, C3H/HeN, C3H/HeJ (LPS insensitive), CBA, C57BL/6 and SCID Beige, representing a variety of genetic and immunologic backgrounds, have been demonstrated to respond to ISS sequences displaying the 5'-PuPu-CpG-PyPy-3' motif (Sparwasser et al., 1997). It has been clearly shown that inclusion of these CpG motifs in plasmid encoded vaccines can, not only ensure the success of the immune responses to the plasmid encoded antigen, but also specifically drive the immune response towards the Th1 phenotype (Raz et al., 1996; Sato et al., 1996; Carson and Raz, 1997; Chu et al., 1997; Roman et al., 1997). Theoretically, direct induction of IFN α and β , IL-12, IL-18, TNF α , in conjunction with indirect NK cell derived IFN γ , could deviate the ensuing immune response, to a plasmid-encoded antigen, to the Th1 phenotype and/or enhance the serum levels of IgG_{2a} (Snapper and Paul, 1987). It has been demonstrated for one protein antigen and a commercially

available subunit human influenza vaccine delivered i.d., that co-delivery of 50 µg of ISSs containing plasmid not only increases total IgG serum levels, but also leads to increases in serum IgG_{2a}, splenic IFN γ and inhibition of serum IgE in BALB/c mice (Roman et al., 1997). Surprisingly, Roman et.al. (1997) also demonstrated that secondary immunization with a DNA-based vaccine could redirect a pre-existing, Ag-specific, Th2 response toward a Th1 type immune response. It has been established that some cytokines, characteristically associated with early innate immune responses (IL-12, IL-18, IFN α and β) are all inducers of IFN γ and promote the development of Th1 lymphocytes (Roman et al., 1997). Thus the ability of the innate immune system to instruct the adaptive immune response, to not only mount an immune response, but to also polarize immunity is potentially useful when protecting against pathogens which require this type of immunity for clearance (Mosmann and Sad, 1996).

It must be pointed out that virtually all studies on ISSs of bacterial origin, or synthetic oligodeoxynucleotides (ODNs), have taken place in mice. Caution, as always, should be exercised when attempting to extrapolate, from ISS data gathered in mice, to other species. For example, the defined, highly efficacious, ISS 5'-PuPu-CpG-PyPy-3" motif described for mice may require unique, alternate flanking sequences to induce immune stimulation in other species. Indeed it has been shown that binding properties of scavenger receptors can differ from species to species (Krieger et al., 1985). For that matter ruminant species, which exhibit a huge gastrointestinal microbial load, may have evolved an innate response that, through necessity, is tolerant, or at least insensitive, to putative ISSs. There is no doubt that ISSs exhibit the potential to play an intriguing role

in ensuring rapid immune responses are evoked against invading pathogens by upregulating many aspects of the innate and, consequently, adaptive immune responses. It may even be possible that this "third genetic code" may define the pre-eminent example of initial early distinction between self and nonself (prokaryote and eukaryote) that drives the decision to mount an adaptive response (Janeway, 1992; Matzinger, 1994; Pisetsky, 1997b).

1.5 Immune Response To DNA-based Vaccines

1.5.1 Intramuscular

There are a growing number of publications describing immune responses to DNA-based vaccines delivered to mucosal surfaces as naked DNA, encapsulated within cationic liposomes, or associated with poly (DL-lactide-co-glycolide) microparticles. However, the next section will focus predominantly on immune responses to DNA delivered i.m., i.d. and ballistically. Table 1.1 shows a compilation of immune outcomes to a variety of DNA-based vaccines delivered i.m. in mice. This is by no means a complete list of references describing immune responses to plasmid encoded antigen injected intramuscularly. However, it is a relatively comprehensive collection of citations which describe data that includes serum IgG isotype and/or splenic or lymph node cytokine profiles in immune mice. This data confirms that mice immunized i.m. with plasmid encoded antigen typically, but not exclusively, respond with a Th1 type immune response, characterized by a predominance of IFN γ , in stimulated splenocytes, and of serum IgG_{2a}. Table 1.1 also shows that there are a significant number of articles that

Table 1.1 Immune Responses in Mice Immunized Intramuscularly with DNA Vaccines

Pathogen or Disease	Antigen + Location	Mouse Strain	Promoter	Method Dose (Boost)	Antibody Response	CMI	References
Bovine Herpesvirus-1	AgD p.m. SgD e.c CgD i.c.	C3H/HeN	RSV	100 ug (Y)	IgG2a>IgG1 IgG1>IgG2a IgG2a>IgG1	splenic IFN γ >>IL-4 " "	(Lewis et al., 1997)
Herpes Simplex Virus-1	gB p.m.	BALB/c	HCMV	90 ug (Y)	IgG2a>IgG1	splenic IFN γ >>IL-4	(Manickan et al., 1995) (Kuklin et al., 1997)
Hepatitis B Virus	pre-S ₂ + S (?)	C57BL/6	HCMV	100 ug (cardiotoxin pretreatment)	IgG1>IgG2a	splenic IFN γ >>IL-4	(Mancini et al., 1996)
<i>M. tuberculosis</i>	Ag85A e.c.	BALB/c C57BL/6	HCMV intronA	100 ug (Y)	IgG1>IgG2a IgG2a>IgG1	splenic IFN γ >>IL-4	(Huygen et al., 1996)
Influenza virus	NP i.c.	BALB/c	HCMV	100 μ g (Y)	IgG2a>IgG1	splenic IFN γ >>IL-4 CTL	(Pertmer et al., 1996)
Influenza virus	HA p.m.	BALB/c	HCMV	100 μ g (Y/N)	IgG2a>IgG1	n.d.	(Deck et al., 1997)

Hepatitis C Virus	(nucleocapsid) /i.c.	BALB/c	HCMV	100 µg (Y/N) Cardiotoxin	IgG2a	splenic IFN γ + IL-2	(Inchauspe et al., 1997)
	C2N (HBV Sag:C fusion)				IgG2a	splenic IFN γ	
Hepatitis B Virus	S (major) pre-S2 (middle)	BALB/c C3H/HeN C57BL/6	HCMV	100 µg (Y/N)	n.d.	splenic IFN γ + IL-2>IL-4	(Chow et al., 1997)
	VP4 i.c.	C57BL/6	HCMV	200 µg (*Y)	IgG after viral boost	splenic IFN γ >IL-4 CTL	(Suradhat et al., 1997)
Influenza A virus	H1 p.m.	BALB/c C57BL/6	?	1,10,25,100 (Y/N)	IgG2a>IgG1	splenic IFN γ >IL-4	(Feltquate et al., 1997)
Experimental Autoimmune Encephalitis	V β 8.2 TCR i.c.	PL/J	HCMV	100 µg (Y) Tibialis and Cardiotoxin pretreatment	IgG1>IgG2a	splenic IL-4>IFN γ CTL	(Waisman et al., 1996)
	circumsporozoite (CSP)	BALB/c	HCMV	40 µg (Y)	IgG1>IgG2a Preboost	splenic IL-4>IFN γ (Preboost)	(Mor et al., 1995)
<i>Plasmodium yoelii</i>					IgG2a>IgG1 Postboost	splenic IFN γ >IL-4 (Post-boost **)	

Herpes Simplex Virus-1	gB	p.m.	BALB/c	HCMV	90 µg (Y)	IgG2a>IgG1	splenic IFN γ>IL-4 +/- CTL	(Manickan et al., 1995)
Measles virus	HA sHA	p.m. e.c.	BALB/c	HCMV	100 µg (Y)	IgG2a>IgG1 but 11 IgG1 with sHA	CTL	(Cardoso et al., 1996)
<i>C. tetani</i>	Fragment C		BALB/c	HCMV	100 µg (Y)	IgG2a>IgG1	splenic IFN γ>IL-5 (check this)	(Anderson et al., 1996)
murine B-cell lymphoma	idiotype		C3H/HeN	HCMV	100 µg (Y)	IgG2a>IgG1	n.d.	(Syrengelas et al., 1996)
Influenza virus	NP i.c.		BALB/c	HCMV	100 µg (Y/N)	IgG2a>IgG1***	splenic IFN γ>IL-4 CTL	(Pertmer et al., 1996)
Hepatitis B virus	S	?	BALB/c	HCMV	5 µg (Y)	IgG1>IgG2a	splenic IFN γ>IL-4	(Gregoriadis et al., 1997)

i.c. intracellular; **p.m.** plasma membrane; **e.c.** extracellular

(?) unknown; (Y) Yes; (N) No; **n.d.** Not done

> greater than; >> much greater than

* boost with DNA at 3 wks, followed by boost with live BRV at 5 wks

** no *in vitro* restimulation of cytokine secreting cells

*** three immunizations with plasmid DNA resulted in decreases in IFN γ and CTL levels, and increases in IL-4 and IgG2a

describe splenic cytokine profiles that do not support the predominance of IgG₁ occurring in the serum (Mor et al., 1995; Mancini et al., 1996; Lewis et al., 1997; Sallberg et al., 1997). Indeed there is at least one article that shows not only a predominance of serum IgG₁ but also levels of IL-4 that exceed those of IFN γ in immune splenocytes (Waisman et al., 1996). These exceptions to the rule of "i.m. delivery of plasmid encoded antigens = Th1-type of immune response", clearly illustrate that parameters such as antigen, strain of mouse, compartment in which the antigen occurs (Cardoso et al., 1996; Lewis et al., 1997) affect the immunological outcome. They also suggest that timing of analysis, presence or absence of secondary immunizations, source of immune cells for ELISPOT (spleen versus draining lymph node versus bone marrow), inclusion or exclusion of an *in vitro* stimulation step and use of ELISPOT or bioassay techniques should be considered when assessing and interpreting immunological outcomes.

There is growing evidence that i.d. delivered DNA is the more efficacious mode of immunization when using DNA-based vaccines. However, there is also recent evidence that incorporation of plasmids encoding antigen(s) within cationic lipids formulated with neutral lipids to form CLDC enhances humoral and T helper cell responses following i.m. delivery (Gregoriadis et al., 1997). Innovations such as these will continue to push the dose and efficacy boundaries until DNA-based vaccines become legitimate alternatives to a variety of conventional vaccines.

1.5.2 Intradermal.

Intradermally injected, and ballistically delivered, DNA-based vaccines target the

immunologically rich dermal and epidermal tissues of the skin. These regions of the murine skin are comprised of a varied population of cell types and include: keratinocytes, endothelial cells, fibroblasts, dendritic epidermal T-cells ($\gamma\delta$ T-cells) and Langerhan cells (Williams and Kupper, 1996). Assumptions are that the epidermal Langerhans cells, with their critical capacity to take up and process antigen, followed by mobilization to regional lymph nodes and subsequent presentation to naive T-cells, are necessary and sufficient for immunity following DNA delivery to the skin. Although epidermal Langerhans cells account for only 1% of skin cells, the surface area available to this relatively small population of these vital cells is in the range of 25% of the epidermal surface area (Abbas et al., 1997). One must also consider the microenvironmental milieu, in the context of injection or ballistic delivery trauma and DNA adjuvancy effects. Keratinocytes, endothelial cells, $\gamma\delta$ T-cells, fibroblasts and mast cells are all capable of responding to trauma or inflammatory molecules by upregulating expression of a variety of cytokines (IL-1 α and β , IL-3, IL-6, IL-7, IL-10, IL-12, IL-15 and TNF α), chemokines (IL-8, MIP 2, IP-10 and RANTES), histocompatibility antigens (MHC I and II), and costimulatory molecules (B7-2), (Bos and Kapsenberg, 1993; Gerritsen and Bloor, 1993; Schroder, 1995; Tigelaar and Lewis, 1995; Williams and Kupper, 1996). This microenvironmental milieu may contribute, in a very significant manner, to the resulting immune character depending on the method of delivery and "adjuvant dose" of DNA. In this way method of delivery and immunological outcome must be considered in the context of the route (skin versus muscle) of delivery.

To date there has been some controversy as to whether i.d. injected DNA elicits a

predominantly Th1/IgG_{2a} response or a Th2/IgG₁ response (Barry and Johnston, 1997; Feltquate et al., 1997). Of the few published articles describing splenic cytokine profiles and serum IgG isotypes there appears to be a split in the immune outcome with antigen, mouse strain, and possibly variations in DNA adjuvancy (dose), playing a greater role than the method, or route, of administration (Table 1.2). Splenic cytokine responses typically suggest that the predominant T helper response is of the Th1 phenotype, although, once again caution must be exercised against over-interpreting this information in the absence of draining lymph node or peripheral blood lymphocyte (PBL) CD4⁺ T-cell cytokine profiles. Typically, potent CTL responses are also achieved following i.d. injection of DNA-based vaccines (Raz et al., 1994). However, none of the authors cited in Table 1.2 assessed this parameter of the immune response.

As seen with i.m. delivered DNA vaccines there does not appear to be a "hard and fast" rule that describes the anticipated serological outcomes to i.d. injected DNA-based vaccines. There does appear to be a tendency towards diminished levels of serum IgG_{2a} as compared to i.m. injected plasmids of the same dose, and this, in conjunction with the suggestive increases in serum IgG₁, supports arguments that the immunological composition of target sites (route) determines to some extent the immune character following delivery of plasmid encoded antigen. Feltquate et. al. (1997), have recently argued that the method (gene gun versus injection) of DNA delivery is solely responsible for the immunological outcome and that the target site cellular composition is essentially irrelevant to the resulting immune character. This data is compelling and it will be of considerable interest and importance to see if this hypothesis holds up with a variety of

Table 1.2 Immune Responses in Mice Immunized Intradermally with DNA Vaccines

Pathogen	Antigen	Mouse Strain	Promoter	Dose (Boost) (Site)	Antibody Response	CMI	References
Influenza virus	NP i.c.	BALB/c	HCMV	100 µg (Y) (Tailbase)	IgG1>IgG2a IgG2a 11 with boost but still < IgG ₁	n.d.	(Pertner et al., 1996)
Influenza A	H1 p.m.	BALB/c	?	100µg (Y/N) (Tailbase)	IgG2a>IgG1 preboost IgG2a>IgG1 postboost	splenic IFN γ>IL-4	(Felquete et al., 1997)
Reporter Gene	β-Gal i.c	BALB/c	HCMV	100µg (Y) (Tailbase)	IgG2a>IgG1	splenic IFN γ>IL-4	(Raz et al., 1996)
B-cell lymphoma	idiotype e.c.	C3H/HeN	HCMV	100µg (Y) (Tailbase)	IgG1>IgG2a	n.d.	(Syrengelas et al., 1996)
Bovine Herpesvirus-1	gD p.m. tgD e.c.	C57BL/6	HCMV intronA	10µg (Y) (Tailbase)	IgG1>IgG2a (gD) IgG1>IgG2a (tgD)	splenic IFN γ>IL-4	(Braun et al., 1997)

i.c. intracellular; **p.m.** plasma membrane; **e.c.** extracellular
(?) unknown; **(Y)** Yes; **(N)** No; **n.d.** Not done
> greater than; **>>** much greater than
11 increase in serum IgG_{2a} levels by 15 fold following boost

antigens and larger selection of mouse haplotypes or in outbred species.

1.5.3 Ballistic.

Ballistic delivery of DNA-based vaccines involves adsorption of small amounts (typically 1.0 µg or less) of plasmid to gold particles and acceleration of the coated particles into the epidermal layer and dermal tissues (Haynes et al., 1996). Originally mylar discs, with DNA+gold particles on one surface, were accelerated onto a stop screen which retained the mylar disc while allowing the gold particles+DNA to continue through the screen and into the skin surface. More recently DNA+ gold particles have been coated on the internal surface of Teflon tubing (Pertmer et al., 1996). One end of the coated tube is placed on the pressure nozzle of the device used to provide propulsion to the particles while the other is placed against the skin surface to be "immunized". Pressurized gas, typically helium, is utilized to accelerate the particles from the sides of the tubing and into the skin. This method of particle delivery minimizes the side spray of the vaccine. Obviously a significant drawback of mylar disc delivery is the potential for waste of vaccine, as well as , the distinct possibility of immunizing, not only vaccinees, but also workers delivering vaccines. Serological responses to ballistically delivered DNA appear to show less variation in isotype than observed in mice immunized i.d. or i.m. (Pertmer et al., 1996; Feltquate et al., 1997). However the current numbers of published articles describing serum antibody isotype and lymph node/spleen/bone marrow B-cell and cytokine profiles is small (Table 1.3) and will demand considerably more studies before any dogmatic statements may be made regarding predilection toward one Th-type or

another. Inclusion or exclusion of secondary immunizations is an important consideration when assessing cytokine or serum isotype data following ballistic immunization with plasmids encoding antigen. Indeed it has been shown that splenic cytokine profiles may show either a predominance of IL-4 or a IFN γ and go on to demonstrate that boosting increases the splenic levels of IL-4 with diminished levels of IFN γ (Pertmer et al., 1996). Conversely, Feltquate et. al. (1997) demonstrated that a splenic Th2 cytokine profile, and accompanying high levels of serum IgG₁, was not altered by subsequent booster immunizations with i.d. or i.m. delivered vaccine. In other words, the priming Th2 response could not be deviated towards the expected Th1 response (with increased levels of IgG_{2a}) when delivering DNA via i.d. or i.m. routes. One might predict that the initial Th2-type response is a direct result of the greatly diminished dose of hypomethylated CpG motif mediated adjuvancy as a result of the dose limits associated with ballistically delivered DNA. Furthermore, one might argue that the trauma experienced by keratinocytes, fibroblasts, Langerhans cells and endothelial cells may create a unique local cytokine and chemokine profile that deviates the developing cognate immune response towards a Th2-type response. However, the contribution of this imagined cellular trauma and postulated inflammatory response may be minimal when one considers that transdermal delivery of liposome packaged recombinant antigen leads to potent Th2 immune responses with virtually exclusive production of serum IgG₁ (personal communication, Dr. M. Baca-Estrada). This immune response to transdermally delivered antigen is restricted to the local draining lymph node and argues that delivery site trauma plays little, or no, role in the ensuing immune character. It also suggests that a

"preset" immune response within lymph nodes draining skin drives the response toward a potent Th2/IgG₁ immunity, in the absence of an immune polarizing adjuvant such as LPS or hypomethylated CpG motifs (Everson et al., 1996; Raz et al., 1996). It would appear that dose, in terms of diminished adjuvancy, and perhaps immune compartmentalization within the immediate draining lymph microenvironment, may be primarily responsible for the Th2-type character of the immune response to ballistically delivered plasmid encoded antigen. Thus, the compelling argument that Feltquate et. al. (1997) have made that the method of DNA delivery determines the immunological outcome must be considered in the context of DNA dose as it affects adjuvancy and immune compartment restriction. Furthermore, the cytokine /chemokine profiles *in vitro*, *ex vivo* and *in situ* following i.d. and ballistic delivery of DNA vaccines must be assessed to ascertain what roles, if any, the route, site or method of DNA delivery has on the developing immune response.

1.6 Mucosal and Epidermal Delivery

1.6.1 Unfacilitated Mucosal Delivery

Administration of DNA-based vaccines to mucosal and skin sites represents an exciting extension to more conventional systemic immunization studies currently underway (Ulmer et al., 1996). Surprisingly, mucosal delivery of naked DNA, without the aid of any cytofectins, results in substantial levels of transfection locally and distally to the inoculation site (Fynan et al., 1993; Etchart et al., 1997; Kuklin et al., 1997). Fynan

Table 1.3 Immune Responses in Mice Immunized with DNA Vaccines Delivered Ballistically

Pathogen	Antigen	Mouse Strain	Promoter	Dose (Boost) (Site)	Antibody Response	CMI ^A	References
Influenza virus	NP	BALB/c	HCMV	100 (Yes) (abdominal epidermis)	IgG1>IgG2a	IFN γ >IL-4 IFN γ >IL-4 * IL-4>IFN γ CTL	(Pertmer et al., 1996)
Influenza A	H1	BALB/c	HCMV	0.04-0.4 μ g (Y/N) (skin)	IgG1>IgG2a	IL-4>IFN γ	(Feltquate et al., 1997)
"	"	"	"	0.04-0.4 μ g (?) (quadriceps)	IgG1>IgG2a	n.d.	"
"	"	C57BL/6	"	0.04-0.4 μ g (Y/N) (skin)	IgG1>IgG2a	n.d.	"

i.c. intracellular; p.m. plasma membrane; e.c. extracellular

(?) unknown; (Y) Yes; (N) No; > greater than; >> much greater than

^A cytokine levels are determined from splenocytes unless otherwise indicated

!! increase in serum IgG_{2a} levels by 15 fold following boost

* successive boosts resulted in decreasing splenic IFN γ and increasing IL-4. After the 3rd boost splenic IL-4 levels exceeded IFN γ levels

et. al. (1993) demonstrated a 75% survival rate against lethal Influenza virus in mice immunized intranasally (i.n.) with 100 µg of a plasmid encoding a hemagglutinin glycoprotein (subtype H1). While mucosal levels of IgA were not assessed it was clearly shown that i.n. immunization primed for systemic IgG responses. Kuklin et. al. (1997) immunized BALB/c mice three times with 100 µg of plasmid encoding glycoprotein B from Herpes Simplex virus (HSV)-1, and demonstrated expression of gB in the lungs and cervical lymph nodes. While these mice developed delayed type hypersensitivity responses and moderate to high levels of IgA in the vagina and feces, they were not protected following intravaginal challenge. Co-administration of 2 µg of cholera toxin (a potent adjuvant) with the DNA vaccine significantly increased the levels of gB-specific IgA occurring at the vaginal mucosa, and offered some protection against a low dose HSV-1 vaginal challenge. Thus the potential of using DNA vaccines at mucosal surfaces has been established. However, researchers recognize the need to investigate methodologies whereby naked DNA may be delivered with greater efficiency to relevant cells associated with mucosal surfaces. Encapsulation of naked DNA within cationic liposomes or microspheres may be two approaches currently available that would enhance delivery of DNA to cells of the mucosa and associated lymphoid tissues.

1.6.2 Liposome Facilitated Delivery

It has been demonstrated that packaging naked DNA vaccines in cationic liposomes or microparticles can greatly increase the efficiency of delivery to certain mucosal surfaces (Puyal et al., 1995; Wheeler et al., 1996; Ban et al., 1997; Etchart et al.,

1997; Gregoriadis et al., 1997; Klavinskis et al., 1997; Wang et al., 1997). Despite preliminary evidence suggesting delivery limitations of liposomes, it now seems apparent that modification of existing cationic and neutral lipids, in conjunction with extensive testing of various combinations of these lipids, can greatly enhance delivery and expression of genes encoded within plasmids. It has been demonstrated that novel cationic lipids such as GAP-DLRIE, and existing cationic lipids such as DOTIM or DOTAP formulated with neutral lipids (DOPE or cholesterol) can enhance *in vivo* uptake and expression of i.n. or i.v. administered cationic liposomes:DNA complexes (CLDC's) 1,000 to 2,000 fold (Wheeler et al., 1996; Liu et al., 1997; Templeton et al., 1997). Immune responses to liposome encapsulated DNA-based vaccines are broad and include distal mucosal antibody and CTL activity, as well as, significant serum levels of IgG and systemic CD8+ CTL activity (Gao et al., 1995; Etchart et al., 1997). These advances in lipid structural modification and formulation, in conjunction with a greater understanding of how the three dimensional structure of cochleates, multilamellar (MLVs), and unilamellar (ULVs), vesicles alter effectiveness of gene delivery, and the potential for targeting to specific cell types predict a significant increase in the success and use of DNA-based immunization (Gould-Fogerite and Mannino, 1996).

1.6.3 Microsphere Facilitated Delivery

Encapsulation or adsorption of plasmids encoding reporter genes or antigens within, or on the surface of, biodegradable microparticles such as poly(DL-lactide-co-glycolide) specifically target and can elicit antigen-specific mucosal IgA responses, as

well as, serum IgG titers (Alpar et al., 1997; Jones et al., 1997). Mice immunized with DNA complexed to microparticles displayed antigen-specific IgA responses at, and distal to, the oral and gastric mucosal administration site. Oral immunization also stimulated good serum IgM and IgG titers. Alpar et. al. (1997) demonstrated that i.n. administration of DNA adsorbed to biodegradable pseudo-polyaminoacid microspheres {poly (DTH carbonate)} induced good systemic IgG responses that equalled, or exceeded, antibody responses to Lipofectin® encapsulated DNA despite using 4-8 times less DNA.

1.6.4 Adenovirus Facilitated Delivery

Co-internalization of replication deficient adenovirus with plasmids encoding genes can increase reporter gene expression in cultured cell lines with efficiencies equal to those achieved with cationic lipid mediated transfection (Yoshimura et al., 1993). Adenovirus facilitated uptake of plasmids encoding gene sequences occurs as a result of receptor mediated binding of virus to ubiquitously expressed receptors on host cells and subsequent endosomolytic activity of viral capsid proteins during acidification of the endosomal compartment (Allgood et al., 1997). Adenovirus mediated escape from the endosomal vesicles leads to the relatively efficient release of other macromolecules within the endosome. A number of modifications to the co-inoculation protocol have been utilized that include: cationic polymer facilitated association of plasmids to adenovirus, cationic lipid and ligand based enhancement of adenovirus vectored plasmid DNA complex uptake (Wagner et al., 1992; Ferkol et al., 1995; Fasbender et al., 1997; Schwarzenberger et al., 1997). Covalent or noncovalent linkage of cationic polymers,

prebound to plasmid DNA, to adenovirus to augment uptake of genes of interest have been reported (Allgood et al., 1997). Typically the cationic polymers have a very high affinity for the polyanionic phosphate groups of DNA and are typically poly-L-lysine or polyethylenimine (Baker and Cotten, 1997). It has been hypothesized that poly-L-lysine sequences may facilitate nuclear targeting of associated DNA sequences (Ferkol et al., 1995). Although receptors for adenovirus are relatively ubiquitous on host cells, researchers have found that *in vivo* transfection of airway mucosa proceeds poorly due to inefficient binding of adenoviruses to the apical surface of epithelial cells (Ferkol et al., 1995). One approach utilized to rectify deficiencies in mucosal epithelial delivery included formulating recombinant adenovirus vectors, expressing β -galactosidase, with polycationic lipids such as Lipofectamine[®], GL-67 or a combination of GL-67/DOPE (dioleoylphosphatidyl-ethanolamine)(Fasbender et al., 1997). Although recombinant viral vectors were utilized in this case it is conceivable that polylysine/adenovirus/DNA conglomerates may be complexed with polycationic lipids to facilitate augmented mucosal delivery, and uptake, of plasmids expressing foreign antigens or immune modulating genes. A second approach utilized to circumvent problems associated with mucosal delivery of plasmids encoding genes involved covalent linkage of Fab fragments, directed against the IgA receptor at the basolateral surface of epithelial cells, to poly-L-lysine and subsequent condensation with plasmid encoding genes of interest (Ferkol et al., 1995). Intravenous introduction of the ligand/poly-L-lysine/DNA complexes leads to specific targeting of plasmids encoding genes to the airway epithelium. Although adenoviruses were not a component of these Fab/poly-lysine/DNA

complexes they have been utilized as ligand-adenovirus-poly-L-lysine complexes and do appear to facilitate receptor mediated augmentation of plasmid delivery to the cytoplasm of specific cell types (Curiel et al., 1991).

1.6.5 Future Prospects

The future of liposomal and microsphere facilitated DNA-based immunization appears to lie in the ability to increase the serum or mucosal half-life of encapsulated DNA and to increase the efficiency of delivery to the cytoplasm of cells. These delivery vehicles also possess the potential to target specific cells of the immune system more effectively than naked DNA alone (Eldridge et al., 1989; O'Hagan et al., 1993) . There is also preliminary evidence that specific cell targeting may be further enhanced through inclusion of appropriate ligands in the outer lipid envelope of liposomal vesicles (Puyal et al., 1995). It is unknown whether targeting will offer any advantages at this point or if this is a possibility with biodegradable microspheres.

1.6.6 Transdermal

The potential exists to immunize animals through topical application of DNA vaccines to the epidermis. The potential of this concept was first demonstrated in 1995 when researchers showed that reporter genes, packaged in liposomes, were transfected into hair follicular cells with high efficiency (Li and Hoffman, 1995). Although there was little evidence for transfection outside of hair follicles, transfection of cells within the base of the hair bulb was clearly evident. Transfection of this cell population, in

conjunction with the vascularization and likelihood of lymphatic drainage of the dermal papillar region allows one to hypothesize that plasmid encoded antigen would quickly gain access to secondary lymphoid tissue. The recent advances in transdermal delivery of drugs and proteins clearly suggests that these advances will be transferred to transdermal delivery of plasmids as well.

1.7 Cytokine Modulation of Immunity to DNA-based Vaccines

Of constant concern to immunologists involved in vaccine development is to ensure that vaccination results in an immune response that best protects animals or humans against a given pathogen or disease (Mosmann and Sad, 1996). In order to accomplish this we must: be able to polarize CD4⁺ T helper cells towards Th2 or Th1 phenotypes, ensure that the magnitude of humoral immunity, when required, is adequate, and finally elicit CD8⁺ T-cell mediated cytotoxicity when protection from infection requires this arm of immunity. DNA-based vaccines currently display the potential to polarize immune responses depending on several parameters, including: form of antigen, method and route of delivery, and dose of plasmid injected (Cardoso et al., 1996; Pertmer et al., 1996; Sato et al., 1996; Feltquate et al., 1997; Lewis et al., 1997; Roman et al., 1997). A further advantage of DNA-based vaccines is the relative ease with which immunomodulating cytokines can be co-administered as plasmid encoded gene(s) (Xiang and Ertl, 1995). The advantages of this approach include: low cost, simplicity of co-delivery, elimination of the need for multiple dosing of recombinant cytokines with short serum half-lives, and avoidance of toxicities and regulatory issues plaguing many current adjuvants (Hughes et

al., 1992; Gupta et al., 1993). Disadvantages of this approach revolve around issues of sustained expression and the potential toxicities (Cohen, 1995) associated with potentially long term responses, as well as the need to identify, isolate, and assess cytokines of importance in target species.

Co-administration of plasmid-encoded cytokines is achieved by delivering a mixture of two separate plasmids, one of which encodes the antigen and one which encodes the cytokine, or by delivering a single bi- or polycistronic vector with internal ribosomal entry sites separating genes representing antigen and cytokine (Clarke et al., 1997). Cytokines may also be delivered as molecular chimaeras comprised of antigen and cytokine usually separated by a domain preserving spacer region (Syrengelas et al., 1996; Maecker et al., 1997). To date a variety of cytokines and growth factors have been co-administered with plasmid encoded antigen and include: GM-CSF, IL-12, IL-2, IL-4, IL-6, IL-7, IL-1 α and β (Table 1.4). Data included in Table 1.4 is not meant to be complete, however, information included summarizes a relatively comprehensive list of publications that have included data describing serum isotype, splenic cytokine profiles and CTL data.

Mice immunized with plasmids encoding antigens and plasmids encoding GM-CSF show substantial augmentation of serum IgG titer, seroconversion efficiency, enhanced cell mediated responses that include blastogenesis and CTL. The mechanisms involved in these responses appear to involve: enhancement of antigen immunogenicity (Iwasaki et al., 1997), significant upregulation of IL-4 in spleens and draining lymph nodes (unpublished data) and the enhancement and maintenance of antigen capture and

processing functions of local, or possibly recruited, dendritic cell populations (Sallusto and Lanzavecchia, 1994; Lutz et al., 1996). Dose, and the presence or absence of secondary immunizations, of plasmids encoding GM-CSF can have very significant, and surprising effects on the immune outcome to antigen co-administered with this cytokine. We were able to demonstrate that a single dose (50 µg) of plasmids encoding GM-CSF, co-delivered with a plasmid encoding a secreted version of bovine herpesvirus (BHV)-1 glycoprotein D (which normally gave us very high serum titers of IgG₁), resulted in suppression of the humoral response (see section 4.3.4). However, post-boost titers reflected a potent anamnestic humoral response and a significant trend towards increased serum IgG_{2a} (see section 4.3.5). Conversely, moderate doses (10 µg) of plasmids encoding GM-CSF showed serum Ig isotypes that were predominantly IgG₁ regardless of whether mice received secondary immunizations. In some instances co-administration of plasmids encoding GM-CSF enhanced antigen-specific cytotoxic immunity. Geissler *et al.* (1997) and Iwasaki *et al.* (1997) were able to demonstrate enhancement of T-cell mediated cytotoxicity to Hepatitis C virus p21 core antigen and Influenza virus nucleoprotein respectively, when plasmids encoding these antigens were co-administered with plasmids encoding GM-CSF.

Co-administered plasmids encoding the p35 and p40 subunits of IL-12 could also enhance T-cell mediated cytotoxicity responses, confer protection against challenge, and deviate immunity towards a Th1-type response (Irvine et al., 1996; Iwasaki et al., 1997; Kim et al., 1997; Tsuji et al., 1997). A decrease in serum IgG levels and splenic, antigen-specific, IL-4 levels were consistent with an increase in cells of the Th1-type

Table 1.4 Immune Responses to Plasmids Encoding Antigen Co-administered with Plasmids Encoding Cytokines

Antigen	Mouse Strain	Cytokine	Dose (Boost) Route/Method	Antibody Response	CMI	References
BHV-1 SgD	C3H/HeN	GM-CSF	10.0 µg (N) i.m.	1 Ab kinetics 1 seroconversion 1 magnitude	n.d.	(Lewis et al., 1997)
BHV-1 SgD 50 µg	C3H/HeN	GM-CSF	2.0 µg (Y) i.m.	1 Ab kinetics 1 magnitude (at 2-4 wks)	1 IL-4 (Spleen/DLN)	(see section 4.3.6)
			10.0 µg (Y) i.m.	1 mean serum IgG (at 2 wks) 1 serum IgG1	1 IL-4 (Spleenic/DLN) 1 IFN γ (Spleenic/DLN)	"
			50.0 µg (Y) i.m.	1 mean serum IgG preboost 1 mean serum IgG postboost 1 serum IgG2a postboost	1 IL-4 (Spleenic/DLN) 1 IFN γ (Spleenic/DLN)	"
Rabies virus G protein 50 µg	C3H/He	GM-CSF	*10.0 µg i.m.	direct correlation between	n.d.	(Xiang and Ertl, 1995)
			*50.0 µg i.m.	dose of GM CSF and mean	n.d.	
			*250.0 µg i.m.	serum IgG titre	n.d.	

20 µg	"	"	100.0 µg (N) i.m.	1 VNA after prime with 11 after successive boosts	n.d.	"
50 µg	"	"	100.0 µg (N) i.m.	no change in survival post challenge	n.d.	"
HIV-1 pEnv	BALB/c	GM-CSF	50 µg (N) i.m.	1 serum IgG	1 blastogenesis n.c. in CTL	(Kim et al., 1997)
pGag/pol	"	"	"	1 serum IgG	11 Blastogenesis, n.c. in CTL	
HCV p21 core protein	BALB/c	GM-CSF	50 µg (Y) i.m.	** 1 seroconversion ** 1 serum IgG titres	n.c. in splenic IFN γ/IL-4 1 CTL but IL-2 + GM-CSF = 11 CTL	(Geissler et al., 1997)
Influenza non immunogenic NP ₀	BALB/c	GM-CSF	100 µg (Y) i.m.	n.d.	11 in CTL after second boost IL-12 + GM-CSF 11 CTL after 1st boost	(Iwasaki et al., 1997)

Carcinoembryonic antigen CEA (2 µg)	C57BL/6	GM-CSF	2.0 µg (Y) ballistic (abd.)	*** ↓ serum titer	*** ↓ blastogenesis	(Conry et al., 1996a)
B-cell tumor idiotype Ag p38c13	C3H/HeN	Id:GM-CSF chimera	100 µg (Y) i.m.	IgG2a>IgG1	n.d.	(Syrengelas et al., 1996)
			100 µg (Y) i.d. (tailbase)	IgG1>IgG2a	↓ survival as compared to rId:GM-CSF conventional vaccine	
BHV-1 SgD (10.0 µg)	C3H/HeN	IL-4	10.0 µg (N) i.m.	↓ mean serum IgG enhanced early humoral kinetics	n.d.	(see section 4.3.4 + 4.3.5)
(50.0 µg)		IFN γ	2.0 µg (Y) i.m.	↓ mean serum IgG ↓ mean serum IgG2a	n.c in splenic IFN γ or IL-4 small ↓ in l.n. IFN γ or IL-4	(see section 4.3.4 + 4.3.5)
			10.0 µg (Y/N) i.m.	↓ mean serum IgG ↓ mean serum IgG2a	n.c in splenic IFN γ or IL-4 ↓ in l.n. IFN γ or IL-4	(Lewis et al., 1997)
			50.0 µg (Y) i.m.	↓ mean serum IgG ↓ mean serum IgG2a	n.c in splenic IFN γ or IL-4	"
(10.0 µg)	C3H/HeN	TNF α	10.0 µg (N) i.m.	↓ 2 wk. Mean serum titers ↓ mean serum IgG2a	n.d.	(see section 4.3.4 +4.3.5)

(10.0 µg)	C3H/HeN	IL-6	10.0 µg (N) i.m.	↓ mean serum IgG enhanced early humoral no impact on early kinetics	n.d.	(Lewis et al., 1997)
**** KLH or Transferrin	BALB/c	IL-2	100 µg (Y) i.m.	↓ serum titer	↓ DTH	(Raz et al., 1993)
		IL-4	100 µg (Y) i.m.	↓ serum IgG ↓ serum IgG1		
plasmid encoded β-gal	BALB/c	IL-2 IL-6 IL-7	1.0 µg (Y) ballistic (Abdominal epidermis)	n.d.	↓ Protection against murine colon adenocarcinoma cell line CT26 transfected with plasmid encoded β-gal IL-2>IL-6>IL-7	(Irvine et al., 1996)
	BALB/c	IL-12	0.1-1.0 µg (Y) ballistic (Abdominal epidermis)	n.d.	↓ Protection IL-2>IL-6>IL-7	
HIV-1 Gag/pol pcENv	BALB/c	IL-12	50 µg (N) i.m. myotoxin	↓ mean serum IgG	↓ Blastogenesis ↓ Spleen size and splenocyte #s ↓ CTL	(Kim et al., 1997)
HIV-1 gp160 rev	BALB/c	IL-12	2-200 µg (N) i.m. (sucrose) (gastrocnemius)	n.d. (?)	↓ DTH ↓ IFN γ, ↓ IL-4 ↓ CTL	(Tsuji et al., 1997)

Influenza Npo	BALB/c	IL-12	100 µg (Y) i.m. Quads	n.d.	CTL post boost co-administration of plasmid encoded B7-2 1's CTL at 1 st boost	(Iwasaki et al., 1997)
Rabies virus G protein	C3H/He	IFN γ	100 µg(?) i.m.	↓ VNA	↓ IL-2	(Xiang and Ertl, 1995)
Hepatitis B virus S or preS ₂ + S	BALB/c	IL-2	10-100 µg (N) i.m. Quads	↓ serum IgG	Blastogenesis IFN γ, IL-2 ↓ IL-4	(Chow et al., 1997)
HCV p21 core protein	BALB/c	IL-2	50 µg (N) i.m. Quads-opposite	↓ serum IgG, ↓ seroconversion efficiency	↓ blastogenesis, ↓ CTL IL-2, IFN γ (Th1 phenotype)	(Geissler et al., 1997)
		IL-4	leg from p21 plasmid injection	↓ serum IgG, ↓ seroconversion efficiency	↓ blastogenesis IL-2, IL-4 (Th0 phenotype)	

(?) unknown; (Y) Yes; (N) No; n.d. Not done; n.c. No change

> greater than; >> much greater than

↓ increase; || Large increase

* = cytokine co-administered at first immunization only

** = not statistically significant

*** = only if plasmid encoded GM-CSF administered 3 days prior to G protein encoding plasmid

**** = antigen delivered as a recombinant purified protein

immune phenotype (Tsuji et al., 1997).

IFN γ is known to be involved in deviation of the immune response towards the Th1 phenotype and the Ig switch to a IgG_{2a} isotype (a surrogate marker for Th1 responses), (Snapper and Mond, 1993). Early published work suggested that co-delivery of plasmids encoding IFN γ resulted in suppression of both humoral and cell mediated immune responses (Xiang and Ertl, 1995). We also found clear evidence for a suppressive humoral effect following co-administration of 10 μ g this cytokine (Lewis et al., 1997). However, we also noted that co-administration of plasmids encoding IFN- γ resulted in increases in relative amounts of IgG_{2a}, although this increase was not consistent in all mice. Finally, we observed that following co-administration of 50 μ g doses of IFN γ , with plasmids encoding a secreted form of BHV-1 gD, the suppressive humoral effect disappeared while the tendency toward increased levels of serum IgG_{2a} was maintained (see sections 4.3.4 and 4.3.5). Interestingly, we found that co-administration of plasmids encoding TNF α , with plasmids encoding a secreted form of BHV-1 gD increased the magnitude of the serum IgG response at 2 weeks and elicited increases in the mean serum IgG_{2a} levels (Lewis et al., 1997). Despite the tendency towards higher levels of serum IgG_{2a} we have not been able to demonstrate consistent isotype switching in individual animals using a dose range of plasmids encoding IFN γ (2 -50 μ g),(unpublished data). Recently, the issue of isotype contribution to protection was raised by Bachmann et. al. (1997), who, using a murine lymphochoriomeningitis virus (LCMV) model, concluded that beyond a threshold level of serum antibody, isotype becomes irrelevant. Although this article is worth noting, it does not diminish the

potential importance of efficient complement fixing isotypes, such as murine IgG_{2a}, in other disease models (Ishizaka et al., 1995), or the protective advantages a predominance of complement fixing isotypes may offer in poor vaccine responders in outbred populations. Thus, it will be of considerable interest to assess other cytokines, such as: IFNs α and β and IL-18, known to play roles in deviating the immune responses towards a Th1-type phenotype and potentially modulate the serum antibody profile, as well as, facilitate the development of cytotoxic CD8⁺ T-cell responses (Roman et al., 1997).

Co-administration of plasmids encoding IL-2 or IL-4 with plasmids encoding antigens, have been demonstrated to enhance humoral and cell-mediated responses (Raz et al., 1993; Chow et al., 1997; Geissler et al., 1997). Mice immunized with plasmids encoding IL-2 and antigen display increases in serum IgG levels, increased seroconversion efficacy, and increased protection following challenge (Irvine et al., 1996). Plasmids encoding IL-2 also appear to enhance: T-cell blastogenesis, cytotoxic T-cell levels, both IFN γ and IL-4 cytokine expression levels within splenic T-cell populations, and increase delayed type hypersensitivity reactions (Raz et al., 1993; Chow et al., 1997; Geissler et al., 1997). We were not able to show any augmentation of immunity following co-administration of IL-2 with a suboptimal dose of plasmids encoding a secreted form of BHV-1 gD. However, we assessed humoral responses only, to a single, moderate, dose of IL-2, that may not have been optimized for C3H/HeN mice (Lewis et al., 1997). Mice immunized with plasmids encoding IL-4 displayed increases in serum IgG in seropositive animals, increases in IgG₁ and seroconversion efficiency (Raz et al., 1993; Geissler et al., 1997). Mice receiving plasmid encoded IL-4 also displayed a

substantial increases in splenic T-cell blastogenic responses following *in vitro* stimulation with antigen. We were able to demonstrate increases in mean serum IgG titers using a 10 µg dose of plasmids encoding IL-4 co-administered with plasmids encoding a secreted form of BHV-1 gD (Lewis et al., 1997).

Several other cytokines including: IL-1 α and β , IL-6 and IL-7 have shown a varying impact on the immune responses in mice immunized with DNA-based vaccines (see Table 1.4). It would appear, at first glance, that these cytokines are not worth pursuing as vaccine modulators, however, it must be pointed out that in many instances responses to a dose range of these plasmids encoding cytokines have not been carried out. In these cases, and with cytokines that do show modulatory effects, dose range impact on immunological outcome must be assessed before any generalizations may be made. Indeed, dose range with each antigen, particularly if the expression compartment varies (intracellular, extracellular etc) in a variety of different murine haplotypic backgrounds, as well as target species, must be carried out before researchers can begin to appreciate the possibilities for immune augmentation or deviation through co-administration of plasmid encoded cytokines.

Finally, several recent articles have assessed immunity to DNA-based vaccines encoding antigen:cytokine fusion proteins (Syrengelas et al., 1996; Maecker et al., 1997). Syrengelas et. al. (1996), demonstrated that i.m. delivery of plasmids encoding a B-cell lymphoma idiotype:GM-CSF fusion protein resulted in significant increases in serum IgG₁ levels that coincided with moderate increases in serum IgG_{2a}. Intradermal delivery of this plasmid resulted in suppression of serum IgG₁, as compared to plasmid encoding B-

cell lymphoma idiotype only. Maecker et. al. (1997), created plasmids encoding fusions between ovalbumin (OVA) and: GM-CSF, IFN γ , IL-2, IL-4 IL-12 and a nine amino acid fragment of IL-1 β known to possess adjuvant activity. Serum antibody levels were minimal with all constructs except for the OVA:IL-4 chimaera. Immunization with plasmids encoding OVA:IL-1 β and OVA:IL-12 fusions appeared to deviate splenic cytokine responses towards a Th1 phenotype and appeared to elicit the strongest T-cell mediated cytotoxic responses. In light of the molecular intricacies involved in creating and expressing stable chimeric proteins, this preliminary data, while novel, must be compared directly to simpler immunization models where modulatory proteins are co-administered as separate plasmids or co-expressed from the same plasmid that encodes antigen.

1.8 Safety

1.8.1 Autoaggression and Autoimmunity

Several safety issues predominate when addressing possible deleterious outcomes to the use of DNA-based vaccines. These issues include: the potential for chronic autoaggression and subsequent development of epitope spreading and the development of anti-DNA antibodies or a Lupus-like syndrome. We have the technology, at least in mouse models, to achieve 25-30% *in vivo* transfection efficiencies following i.m. immunization (Hartikka et al., 1996). Several recent articles demonstrating the existence of CTL activity within the muscle mass (Davis et al., 1997; Yokoyama et al., 1997), clearly illustrate the potential for significant levels of immune mediated myositis and

potentially the development of a true autoimmune disorder through epitope spreading (Vanderlugt and Miller, 1996). While the development of autoimmunity through epitope spreading remains a possibility there has been at least one published instance where assessment of immunity towards the self antigen myosin failed to detect any evidence autoimmune responses following i.m. DNA immunization (Mor et al., 1997).

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by substantial levels of serum IgG that are typically reactive to double stranded mammalian DNA (Gilkeson et al., 1989; Steinberg et al., 1990). Gilkeson et. al. (1989) demonstrated that immunization of normal mice with bacterially derived (*Escherichia coli*) DNA, complexed to methylated bovine serum albumin (mBSA) in Freund's complete adjuvant (CFA) elicits very high levels of anti-bacterial DNA antibodies. However, these mice showed no evidence of crossreactivity with mammalian (calf thymus) dsDNA. More recently it was demonstrated that immunization of BALB/c mice with single stranded DNA complexed with mBSA in CFA also induced significant renal pathology (Gilkeson et al., 1993). Most recently, crossreactive anti-mammalian dsDNA antibodies developed in preautoimmune NZB/NZW mice immunized with bacterial DNA in mBSA and CFA (Gilkeson et al., 1995). Thus it is possible, using heavily adjuvanted bacterial DNA, to elicit anti-DNA antibodies. However, responses require formulation with potent adjuvants, appear to be crossreactive only in susceptible strains of mice and renal injury appears to require ssDNA of bacterial origin as the immunizing antigen. There remains the possibility that the potent immunostimulatory sequences encoded within injected plasmids may provide sufficient costimulatory signalling to activate B-cells

simultaneously recognizing plasmid DNA through engagement at the B-cell receptor (Krieg et al., 1998). Certainly it has been clearly demonstrated that immunostimulatory sequences within plasmid DNA can induce proliferation, MHC class II antigen expression, costimulatory signal upregulation and immunoglobulin synthesis in naive B-cells.

Although it is still early, there has been no evidence for the sustained development of anti-DNA antibodies in animals immunized with naked DNA-based vaccines (Mor et al., 1997). There has been some suggestion of a transient, predominantly IgM, antibody response. However, this response did not develop into a clinically relevant problem. Researchers investigating the potential of DNA-based vaccines to cause a Lupus-like syndrome have focussed on assessment of humoral responses to DNA. Recent information indicates that autoimmune antibodies characteristic of this disorder are not directed solely against nucleic acids (Speciale, 1993). In over 95% of persons with systemic lupus erythematosus (SLE) antibodies were found against a 30 kDa cell surface protein that binds cell membrane DNA (Bennett et al., 1987; Bennet et al., 1988). The role these anti-DNA receptor antibodies play in the development or maintenance of SLE is unknown too date. Indeed, these antibodies may simply be a diagnostic indicator for an increased risk of development of SLE or may be involved in the conversion of the transient, T-cell independent, IgM response to a T-cell dependent response. In either case, it will be important to screen for seroconversion to this neoantigenic complex (plasmid DNA:30 kDa receptor) when studying the potential for development of autoimmune disorders resembling systemic lupus erythematosus following immunizations with DNA-

based vaccines.

1.8.2 Integration

Integration of injected plasmid DNA into host genomic sequences remains an issue of contention between researchers and regulatory agencies (Robertson, 1994). Initially, when it was felt that the myocytes were unique in the ability to take up plasmid DNA and translocate it to the nucleus, the argument was made that the absence of mitotic activity in myocytes would virtually eliminate the likelihood of hazardous mutational integration occurring (Wolff et al., 1992; Danko and Wolff, 1994). Of course the frequent use of myotoxic agents such as cardiotoxin, or bupivacaine, to upregulate myoblastic mitotic activity diminished the importance of this argument. Similarly, the demonstration that uptake and nuclear localization can occur in a variety of cell types, and that injected DNA does not remain immediately adjacent to the injection site has raised the possibility of genomic integration once again ((Raz et al., 1994; Condon et al., 1996; Torres et al., 1997). More recently, using i.m. injection of plasmids at doses of 100 µg per quadriceps, researchers were unable to demonstrate any evidence of integration in the genomic DNA harvested from 11 tissues (Nichols et al., 1995). Indeed these authors calculated that a mutation event arising from random integration of plasmid DNA into a host gene is 1.3×10^{-9} /cell. However, mutational events do not necessarily require integration into a host cell gene to result in a deleterious outcome, and with a spontaneous random integration frequency calculated at 2×10^{-6} , one may assume that the mutational frequency might actually be higher than the 1.3×10^{-9} /cell value given. To the authors credit, they

envisioned a "worst case scenario" and assumed each "gene" was comprised of 1.0×10^6 base pairs, an excessive length that would allow for integration into intron, regulatory or other sequences not immediately adjacent to the coding region of the "gene". Indeed, the value these authors arrived at may actually be artificially high.

Integration into the genomic DNA of stem cells, particularly those of the reproductive organs, have been areas of concern, particularly in light of growing interest in liposomal or microsphere mediated delivery and the circumvention of many of the uptake limitations that occur when delivering naked DNA.. However, liposome packaged, i.v. administered, plasmid DNA has been demonstrated to partition poorly to the testes or ovaries of mice (Lew et al., 1995). This evidence, although speculative, to some extent, suggests that the injection of DNA as a systemic or mucosal vaccine in animals and humans does not provide great risk to recipients. Conversely, there is recent evidence demonstrating unequivocally that integration can occur following intrasplenic or oral administration of naked plasmid DNA (Gerloni et al., 1997; Schubbert et al., 1997). Certainly this may be an unfair assessment of the integration potential of DNA vaccines because it has been demonstrated that the Ig heavy chain switch region of B-cells are high frequency insertional sites for transfected DNA (Baar and Shulman, 1995). However, these results suggest that integration is a very real possibility and, more importantly, describe a procedure where high frequencies of integration can occur. This data will allow researchers to begin to determine what the integration potential is for a given plasmid delivered in a specific manner and perhaps provide the tools and information to establish assessment criteria that will determine the true risk of genomic integration into relevant

and irrelevant host cells. It would be unfortunate to have to assess the integration potential of DNA-based vaccines on a plasmid by plasmid basis as sequences within DNA-based vaccines are altered to accommodate changes in regulatory elements or new antigens. This "overkill" approach for determining risk assessment of DNA-based vaccines would be unfortunate and, in all likelihood, unnecessary, particularly in light of the fact that a nonprescription form of genomic DNA (from salmon sperm) has been taken by injection, topical administration and orally by people for decades without any apparent adverse effects (Whalen and Davis, 1995). Indeed the oral doses of this DNA-based supplement are 1000-100,000 fold greater (125-250 mg) than doses injected into mice . Of course there remains fundamental differences between intrinsically immunosuppressive eukaryotic DNA and, profoundly immunostimulatory bacterial DNA that must not be overlooked. Indeed, short linear fragments of self DNA have been identified that are normally bound to the outer cell membrane of antigen presenting cells (Lerner et al., 1971). The exact function of this cell surface population of nucleic acid is unknown, however, it is possible these sequences serve to suppress effector T-cell functions that may be directed against antigen presenting cells presenting antigen in the context of major histocompatibility antigens (Russell and Golub, 1978). This information, in the context of mucosal bacterial loads and the resulting exposure of host tissues to both genomic and extrachromosomal DNA, suggests that risks of deleterious mutation events, due to integration of nonself DNA, at mucosal surfaces is minimal. Finally, for the food animal industry the question of integration may be moot, given the time that these animals would be exposed to any potential deleterious effects resulting from a

integration/mutation event following DNA immunization.

1.9 Future Directions

1.9.1 Mechanistic Studies

Poor understanding of the exact mechanism of immune induction by DNA-based vaccines necessitates the need for identification of relevant cell populations involved in immune responses and determination if naked DNA uptake by cells is a receptor mediated phenomenon. Identification of critical cell populations and an uptake mechanism will potentially allow scientists to direct plasmids encoding antigens or immunomodulatory genes to cell populations in a more efficient and specific manner.

Immunostimulatory sequences, or hypomethylated CpG motifs have a profound impact on the immune outcome to DNA vaccines and should prove to be a very interesting area of study. There are several issues with regard to immunostimulatory sequences that remain to be resolved and include the question of adjacent High-G sequences and their putative involvement in surface receptor binding and uptake. One may predict that motifs such as the High-G sequences may be playing a fundamental role in facilitating uptake of injected plasmid DNA, not only for adjuvant effects but also for translocation to the nucleus. Identification and mutation of these could possibly enhance natural transfection efficiency, targeting to specific APC populations as well as more efficacious adjuvant effects.

1.9.2 Target Species

Table 1.5 summarizes delivery, dose, immunological and challenge outcomes for a variety of target species. Generally speaking, the preliminary data summarized in Table 1.5 suggests that there is a very real potential for DNA-based vaccines to offer protection against diseases in outbred populations of veterinary importance. Efficacy of DNA vaccines has been a concern particularly when faced with "scale-up" to species, such as cattle, that are 2,000 times heavier at birth than the typical adult laboratory mouse.

Immunization of cattle with plasmids encoding the bovine herpesvirus 1 glycoprotein D (BHV-1 gD) required substantial doses of DNA with multiple boosting when administered i.m. (Cox et al., 1993). Resulting serum antibody titers were relatively low when vaccine was delivered by this route although moderate levels of protection did occur. Subsequently it was demonstrated that i.d. administration of plasmid encoding a secreted version of BHV-1 gD (tgD) in the ear of cattle elicited moderate doses of serum antibody and offered greater levels of protection than immunization with plasmids encoding the membrane anchored authentic version of BHV-1 gD (van Drunen Littel-van den Hurk et al., 1998). Additionally, immunization in the dermis of the ear in these cattle focusses immune responses within the parotid lymph node which also drains the external nares and eyes in cattle (Sisson and Grossman, 1963). Localizing immunity within this lymph node will likely contribute to the level of local protective immunity at the appropriate mucosal route of entry for BHV-1 (Gao et al., 1995). In this instance it appears that choice of route and method of immunization had a

Table 1.5 DNA-based Vaccination in Target species

Target Species	Pathogen/Antigen	Method/dose Boost	Immune Response	Challenge Outcome	References
Cattle	BHV-1 gD	i.m./500 µg or 125 µg 4 boosts	moderate serum ELISA and s.n. Ab at 20 weeks in high dose animals low Ab in Low dose animal	decreased clinical signs decreased viral shedding	(Cox et al., 1993)
Cattle	BHV-1 gD and *IgD	i.m./1 mg 2 boosts i.d. (ear)/500 µg 1 boost	low ELISA and s.n. Ab moderate ELISA and s.n. Ab	n.d. with i.m. vaccinees decreased clinical signs decreased viral shedding IgD offered greater protection	(van Drunen Littel-van den Hurk et al., 1998)
Cattle	BRSV G protein	i.d. + i.m. total of 1.0 mg multiple boosts	moderate to high serum ELISA Ab	decreased viral shedding at day 7 but not at day 5	(Schrijver et al., 1997)
Cattle	<i>Theileria annulata</i> Tams 1-1,1-2	i.m./ 500 µg/of each antigen 2 boosts	no serum ELISA Ab	50-60% decrease in mortality following lethal challenge	(d'Oliveira et al., 1997)

Sheep	<i>Taenia ovis</i> 45W	i.m./200 µg 2 boosts	low serum ELISA Ab with DNA only	n.d.	(Rothel et al., 1997)
		i.d./200 µg 1 boost	moderate serum ELISA Ab with DNA followed by rec45W/QuilA boost		
Pigs	PRV gD	i.m./400 µg 1 boost	no serum ELISA or s.n. Ab preboost Low serum ELISA and s.n. Ab post boost	no significant clinical protection	(Monteil et al., 1996 Pigs PRV gD i.m./370 mg/l boost with commercial vaccine low s.n. Ab preboost increases in s.n. Ab postboost highest level of clinical protection as compared to a variety of vaccination regimes [Le Potier, 1997 #518])
Poultry	Influenza H1, H7	i.m., i.v., i.n., i.n./100-200 µg 1 boost	n.d.	increased protection in 25-63% chickens	(Pynan et al., 1993)

Ducks	DHBV pre s/s and S protein	i.m./250 or 750 µg 2 boosts	high serum ELISA Ab after 3 rd immunization	increased rate of systemic viral clearance in animals immunized with S protein reduced viral replication in hepatocytes increased <i>in vivo</i> neutralization with anti-S sera but not with anti pre-S/S	(Triyathni et al., 1998)
Fish	IHNV NP and G protein	i.m./10 µg	increased serum ELISA Ab at 8 wks. with G protein and at 4 wks. with G protein + NP increased s.n. Ab at 6 wks with G protein and G protein + NP	50-60% decrease in mortality following lethal challenge	(Anderson et al., 1996)
Cats	FIV env	i.m./400 µg 2 boosts	low to undetectable serum ELISA Ab	enhancement of infection	(Richardson et al., 1997)
Dogs	IL-2, IL-6, GM-CSF	ballistic/0.5 µg buccal mucosa or epidrmlis	increased infiltration of neutrophils at epidermal injection site with GM-CSF no change at buccal mucosa	n.d.	(Keller et al., 1996)

Rabbits	CRPV major capsid protein (L1)	ballistic/1.0 µg over 30 sites 3 boosts	high serum ELISA and s.n. Ab	90-100% protection following challenge	(Sundaram et al., 1997)
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antigen-specific
proliferation of PBMC's

i.m. intramuscular; **i.d.** intradermal; **i.n.** intranasal; **i.t.** intratracheal; **n.d.** not done; **s.n.** Serum neutralization
 * truncated, secreted version of bovine herpesvirus-1 glycoprotein D

profound impact on the immunological outcome. Although there is the suggestion that delivery to dermal tissue results in greater immunological efficacy, the potential for delivery to i.m. tissues must not be ignored. Indeed it has been demonstrated that DNA immunization of different muscle groups within mice can result in substantial differences in the magnitude of the immune responses (Yokoyama et al., 1997). It is also important to recognize that humoral immunity (levels of serum antibodies) are not always a good indicator of protection against a given disease. Intramuscular immunization of calves with plasmids encoding protective antigens from the parasite *T. annulata* failed to elicit detectable serum antibodies and yet provided significant levels of protection in 66% of animals following lethal challenge (d'Oliveira et al., 1997). Similarly, dogs immunized with plasmids encoding canine parvovirus antigens did not demonstrate any antibody but were still protected from parvovirus infection (Schultz, R., personal communication).

In two instances there was either no evidence of protection (Monteil et al., 1996) or disease progression was actually exacerbated following immunization (Richardson et al., 1997). In one instance one day old neonatal pigs from nonimmune mothers were immunized i.m. with plasmids encoding glycoprotein D from Pseudorabies virus and failed to develop detectable levels of serum antibodies (Monteil et al., 1996). In this case immaturity of the neonatal immune system was likely a complicating factor.

Immunization of young cats with plasmids encoding the Feline Immunodeficiency virus envelope protein resulted in enhancement of the early stages of infection (Richardson et al., 1997). In this instance, the authors felt that low serum antibody titers or low affinity antibodies may have played a role in dissemination of challenge virus, or that antigen,

superantigen, or plasmid derived immunostimulatory sequence mediated activation of T- or B-cells that may have augmented early levels of viral replication.

Preliminary data supports the potential for efficacy of DNA-based vaccines in target species. Enhancement of DNA vaccine efficacy in these species will necessitate a closer look at: the method of immunization, targeting of appropriate immunization sites, the appropriateness of antigen, expression cassette choice, and identification of the most effective cellular compartment in which antigen expression occurs. Co-administration of plasmids encoding cytokines is an area that has been largely unexplored in target species, as has enhancement of plasmid uptake by microparticle, adenoviral or liposomal facilitated delivery. These methods, and others, offer mechanisms whereby immune responses to DNA-based vaccines, in target species, can be made more efficacious and practical.

1.9.3 Neonatal Vaccines

Presence of maternal antibody in the sera of neonates specifically inhibits the development of humoral responses in vaccinated animals (Ravetch, 1997). Conventional neonatal vaccination typically involves multiple high-dose immunization regimes designed to minimize the time between waning passive immunity and development of active immunity during which young animals are susceptible to disease (MacDonald, 1992). For many species of food animals this approach may not be cost effective, or achievable, within current management practices.

Use of DNA-based vaccines may allow certain advantages over existing licensed

conventional vaccines when vaccinating neonatal animals of immune mothers. First longevity of expression of these vaccines may facilitate a more efficacious method of immunization such that all animals in a litter, or herd develop active titers as quickly as possible after suppressive maternal derived serum titers are no longer protective (Wolff et al., 1992). Second, DNA-based vaccines almost invariably induce potent CTL and frequently Th1-type immune responses and may prove to be an alternative, significantly advantageous, objective in neonatal animals.

At this point it is presumptuous to assume that longevity of expression afforded by DNA-based vaccines will outperform existing or candidate adjuvants currently available (Dalsgaard et al., 1990; Cox and Coulter, 1997; Davis et al., 1997; Yokoyama et al., 1997). This may certainly be the case when protection to a given pathogen requires a potent neutralizing antibody response. Consideration must also be given to the issue of immunostimulatory sequences within DNA-based vaccines and the contribution these sequences are expected to make to the immune response (Roman et al., 1997). Certainly passively acquired maternal antibodies may serve to functionally separate the benefits of the adjuvant effect of CpG motifs within delivered plasmids and the time point at which antigen interacts with naive B-cells without the simultaneous, inhibitory, co-ligation of the FcγRIIB1 receptor. This concept is further complicated when using neonatal mice as models for investigating this phenomenon due to recent evidence that the naivete of the immune system in this species is such that high zone tolerance, or a profound deviation towards a Th2-phenotype, is the outcome to standard adult doses of vaccine (Forsthuber et al., 1996; Ridge et al., 1996). Indeed, at least one recent article demonstrated B and T-

cell tolerance in neonatal mice following an i.m. (gluteal) immunization with a low dose (10 µg) plasmid encoding the circumsporozoite protein (Mor et al., 1996). These problems, and others, will challenge the ability of any vaccine to elicit strong B-cell mediated immunity in passively immune neonatal animals. Fortunately, DNA-based vaccines have the unique capacity to activate the cell-mediated and cytotoxic arms of the immune response and indeed, may be a more appropriate and achievable goal with many pathogens. Obviously, the presence of a primed expanded CTL population would convey a significant degree of protection to a variety of pathogens (Mosmann and Sad, 1996). Furthermore a primed population of Th cells would narrow the window of susceptibility to disease by shortening the time to anamnestic immune responses. To date, no published article has described the development of humoral immunity in neonate models. However, clear evidence of CD4⁺ T-cell priming and development of CD8⁺ cytotoxic T-cells has been described (Bot et al., 1996; Hassett et al., 1997; Wang et al., 1997). Obviously duration of antigen expression is also a determining factor and will depend, to a large extent, on the species studied, antigenicity, method and route of immunization. This aspect of DNA-based vaccines and the growing awareness that plasmid uptake and expression is occurring within critical lymphoid compartments distal to the site of vaccination make the largely unexplored area of DNA-vaccine applications to neonatal immunity an exciting area of research.

1.9.4 Identification and Manipulation of Novel Antigens

In 1995 a group of researchers described a technique, termed expression library

immunization which provided new possibilities for rapid, yet exhaustive, identification of protective immunogens from a variety of pathogens (Barry et al., 1995). They created an expression library that represented many of the antigens from the lung pathogen *Mycoplasma pulmonis* and immunization of mice with these expression libraries elicited immune responses. While this approach established that specific identification of antigens was not necessarily required for creation of a protective immune response, it did provide the basis for an unconventional, yet highly efficient, procedure to screen entire genomes of pathogens for protective epitopes. Researchers involved in these studies have projected that expression library creation and initial screen for immunogens would take between 6 and 12 months for most pathogens (Taubes, 1997). Indeed, they have currently accomplished this task for several bacteria including *M. pulmonas* and *Mycobacterium tuberculosis* (Taubes, 1997). There are several potential caveats inherent in this technique that include: antigenic competition and partial protein expression resulting in the loss of conformational epitopes (Ulmer and Liu, 1996).

Ultimately DNA-based vaccines are faced with many of the problems that have historically plagued immunologists involved in conventional vaccine development. Perhaps the most relevant problem involves poor antigenicity. Ultimately if a given antigen tends to elicit poor immune responses as a conventional vaccine it may also work poorly as a DNA-based vaccine. This leaves researchers with no choice but to spend money, time and effort in dissecting the structural and functional characteristics of the antigen such that an informed, logical approach can be developed to enhance the antigenicity of the protein in question.

Bovine, human and murine rotavirus VP7 antigen is a case in point. This antigen

has been notoriously difficult to elicit immune responses against despite evidence that protective responses directed against VP7 are protective (Andrew et al., 1990; Hermann et al., 1996). Researchers have utilized a variety of different approaches to enhance the immunogenicity to these antigens with varying degrees of success. We have demonstrated that plasmids encoding bovine rotavirus VP7, delivered by a variety of different routes and methods, fails to elicit either humoral or cell-mediated immunity (unpublished data). Attempts to enhance the immunogenicity by altering the cellular compartment in which VP7 normally occurs and by the addition of transmembrane anchoring domains from BHV-1 enhanced *in vitro* expression but failed to elicit immune responses following immunization. Co-administration of plasmids encoding GM-CSF and IL-4 also failed to facilitate immunity to this antigen (unpublished data). Finally, fusion of VP7 to multiple copies of C3d fragment, shown to be a potent adjuvant for B-cell response, did nothing to enhance immune responses to VP7 despite evidence showing increased expression *in vitro* and greater stability of expression product (Dempsey et al., 1996). We are faced with the necessity to dissect this antigen in an effort to determine if peptide sequences exist that specifically inhibit the responses to this antigen (Ijaz et al., 1991; Volchikov et al., 1992; Hengel and Koszinowski, 1997). Fortunately, DNA-based vaccine technology provides the methodology to significantly shorten the time and cost required for preliminary testing of VP7 subfragments. In this instance the obvious value of expression library immunization for the rapid identification of a strong immunogen, and the strength of the DNA-based vaccine approach to rapidly screen candidate isolates becomes self-evident.

1.10 Summary

Therapeutic and prophylactic DNA vaccine clinical trials for a variety of pathogens and cancers are underway (Chattergoon et al., 1997; Taubes, 1997) . The speed with which initiation of these trials occurred is no less than astounding; clinical trials for an HIV gp160 DNA-based vaccine were underway within 36 months of the first description of "genetic immunization" (Tang et al., 1992) ,and within 24 months of the first published article describing i.m. delivery of a DNA vaccine (Ulmer et al., 1993).

Despite the relative fervour with which clinical trials have progressed, it can be safely stated that DNA-based vaccines will not be an immunological "Silver-bullet". In this regard, it was satisfying to see a recent publication entitled "DNA Vaccines-A Modern Gimmick or a Boon to Vaccinology?" (Manickan et al., 1997). There is no doubt, that this technology is well beyond the phenomenology phase of study. Research niches and models have been established and will allow the truly difficult questions of mechanism and application to target species to progress. These two aspects of future studies are intricately interwoven and will ultimately determine the necessity for mechanistic understanding and the evolution of target species studies. The basic science of DNA vaccines has yet to be clearly defined and will ultimately determine the success or failure of this technology to find a place in the immunological arsenal against disease.

In an recent commentary article by Ronald Kennedy (1997) in which he discusses and introduces a research article describing protection against heterologous challenge with HIV-1 in chimpanzees he begins his article by stating, "As someone who has been in the trenches of AIDS vaccine research for over a decade and who, together with collaborators, has attempted a number of different vaccine approaches that have not

panned out, I have a relatively pessimistic view of new AIDS vaccine approaches". Kennedy goes on to describe a DNA-based, multigene vaccine approach and the subsequent development of neutralizing titers and potent CTL activity in immunized chimpanzees (Boyer et al., 1997). Dr. Kennedy closes his commentary by stating, "The most exciting aspect of this report is the experimental challenge studies.....Viraemia was extremely transient and present at low levels during a single time point. These animals remained seronegative ...for one year after challenge", " Overall, these observations engender some excitement " (Kennedy, 1997). While this may seem less than a rousing cheer for DNA-based vaccine technology it is a refreshingly hopeful outlook for a pathogen that experience has taught us humility. It has also been suggested that DNA-based vaccine technology may find its true worth as a novel alternate hope for development of vaccines for diseases that conventional vaccines have been unsuccessful in controlling (Manickan et al., 1997). A difficult task for any vaccine let alone a novel technology. DNA-based vaccine technology represents a powerful and novel entry into the field of immunological control of disease. The spin-off research has also been dramatic and includes: the rediscovery of potency of bacterially derived DNA sequences (Gilkeson et al., 1989) , and availability of a methodology that allows extremely rapid assessment and dissection of both antigens and immunity. The full utility of this technology has not yet been realized and yet the broad potential is clearly evident. Future investigations of this technology must not be hindered by impatience, misunderstanding, lack of funding or failure of an informed collective and collaborative effort.

2.0 OBJECTIVES

DNA-based vaccines represent a recent, and novel, technological advancement in the field of vaccine development. These vaccines have been demonstrated to deviate immune responses in a manner dependent on: method or route of delivery, type of antigen encoded and identity of co-administered immunomodulatory molecules. Mice and cattle develop humoral and cell-mediated immunity to plasmids encoding glycoproteins B, C and D from bovine herpesvirus (BHV)-1, an economically important pathogen in cattle. However, details regarding the character of the immune responses to these plasmid encoded antigens are limited. Furthermore, the full utility of DNA-based vaccine technology to modulate immunity by co-administration of plasmids encoding cytokine(s) and antigens has only recently begun to be explored. During his graduate program, Dr. Graham Cox, initiated studies on DNA-based vaccines using several plasmid encoded antigens from BHV-1. This was one of the early reports that plasmid based vaccines could induce immunity in animals. Despite this compelling evidence this novel vaccine technology has been greeted with skepticism by many involved in vaccine development and immunity. We hypothesize that immune responses to DNA-based vaccines represent a very real immunological phenomenon. The studies in this thesis are directed toward the characterization of immune responses in mice to DNA vaccines with the following specific aims:

(i) Create plasmids that efficiently express plasma membrane anchored, secreted and intracellular versions of BHV-1 gD.

(ii) Immunize C3H/HeN mice i.m. with each of these DNA vaccines and determine the efficacy and character of immune responses.

(iii) Create expression plasmids encoding each of eight different murine cytokines (IL-1 α , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN γ , TNF α).

(iv) Characterize immune responses in C3H/HeN mice following co-delivery of plasmids encoding these cytokines with plasmids encoding a secreted form of BHV-1 gD.

(v) Characterize the immune responses in passively immune mice (C3H/HeN and C57BL/6), following immunization with DNA and conventional vaccine formulations.

3.0 IMPACT OF ANTIGEN COMPARTMENTALIZATION ON THE IMMUNE RESPONSE IN C3H/HeN MICE RECEIVING DNA-BASED VACCINES

3.1 Introduction

Bovine herpesvirus -1 is a member of the subfamily Alphaherpesvirinae and is the causative agent of infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) (Roizman et al., 1981; van Drunen Littel-van den Hurk et al., 1993a). BHV-1 has also been described as a component of the etiology of certain conjunctival infections, abortions, meningoencephalitic diseases and infectious balanoposthitis (Gibbs and Rweyemamu, 1977; Wyler et al., 1989). Finally, it has been suggested that BHV-1 may be the primary viral agent involved in the development of secondary opportunistic bacterial infections leading to "shipping fever" in cattle (Yates, 1982; Tikoo et al., 1995). Of the 13 proteins associated with the viral lipid envelope glycoproteins B (gB), C (gC) D (gD) are consistently recognized by convalescent sera from BHV-1 infected animals (Collins et al., 1985; van Drunen Littel-van den Hurk and Babiuk, 1986). Immunization of cattle with each of these individual glycoproteins, formulated with a conventional adjuvant, leads to protective immune responses that include: neutralizing serum antibodies and cell-mediated immunity (CMI) (Babiuk et al., 1987; Hutchings et al., 1990; van Drunen Littel-van den Hurk et al., 1993b; Tikoo et al.,

1995). Immunization of cattle with gD typically results in humoral responses of the greatest magnitude and most consistent CMI (Babiuk et al., 1987; van Drunen Littel-van den Hurk et al., 1993b). Also, gD has been demonstrated to efficiently induce CMI and humoral immune responses in C57BL/6 (H-2^b) mice following immunization in a novel water-in-oil adjuvant (Baca-Estrada et al., 1996). Finally, it was previously demonstrated that a DNA-based vaccine, encoding the authentic, membrane anchored versions of gB, gC and gD, efficiently induced humoral immune responses in BALB/c (H-2^d) mice and cattle (gD only) (Cox et al., 1993).

DNA immunization, also termed Polynucleotide, Nucleic acid or Genetic immunization, represents an exciting, novel approach to eliciting protective immune responses in animals (Tang et al., 1992; Donnelly et al., 1993; Ertl and Xiang, 1996; Hassett and Lindsay Whitton, 1996; Ulmer et al., 1996a). This "third revolution" in vaccinology involves the introduction of naked, bacterially derived, plasmid DNA carrying eukaryotic gene regulatory elements driving the expression of genes encoding antigens (Waine and McManus, 1995). Delivery of these DNA-based vaccines involves direct injection of plasmid DNA solubilized in saline or ballistic delivery of gold or tungsten microparticles coated with DNA (Haynes et al., 1996). Injection of DNA is typically intramuscular or intradermal (i.m. or i.d., respectively), while a "gene gun" delivers DNA directly to epidermal or intradermal sites. More recently, DNA encoding antigens or reporter genes have been delivered topically (epidermis) and mucosally (respiratory) in a number of species (Stribling et al., 1992; Li and Hoffman, 1995; Wheeler et al., 1996). The mechanism whereby these naked DNA-based vaccines lead to

highly efficacious immune responses is slowly being unravelled, however, somatic cell uptake of DNA, followed by expression of encoded antigen is a fundamental feature of DNA-based immunization (Raz et al., 1994; Condon et al., 1996; Corr et al., 1996; Ulmer et al., 1996c). Antigen presentation by transiently transfected, professional antigen presenting cells (APC), or indirect presentation following acquisition of antigen from nonprofessional APC by professional APC, is a crucial component of the process (Raz et al., 1994; Condon et al., 1996; Corr et al., 1996; Ulmer et al., 1996c). Recently, it has been demonstrated that bacterially derived DNAs exhibit a potent adjuvant effect that is a function of hypomethylated CpG motifs (Pisetsky, 1996a; Pisetsky, 1997a). Regardless of the mechanistic hurdles remaining it is evident in light of the significant numbers and diversity of pathogens that have been immunized against and animal species that have been immunized that this method of immunization shows considerable promise.

Another significant feature of DNA-based vaccines is the unique ability to activate both humoral and cell-mediated immune responses in vaccinated animals. It has also been stated that not only does the CMI manifest as potent CD8⁺ facilitated cytolytic activity but the predominant CD4⁺ T helper (Th) subset is comprised of interferon (IFN)- γ secreting Th1 cells (Ulmer et al., 1996a). T helper cells have been classified into several major groups that include: Th1, Th2 and Th3 (Mosmann et al., 1986; Chen et al., 1994). Th1 cells are characterized by secretion of interleukin (IL)-2, IFN- γ and tumour necrosis factor (TNF)- β . Th2 cells are characterized by secretion of interleukins 4, 5, 6 and 10, while Th3 cells are proposed to be a suppressor population expressing transforming growth factor (TGF)- β , IL-10 and IL-4. DNA-based vaccines have been

suggested to be of greater benefit than many conventional vaccine formulations because they induce a predominantly Th1-type of immune response that would be a more appropriate protective immune response against a variety of pathogens (Fresno et al., 1997).

Of course humoral immunity also plays a significant role in prevention of pathology and it has been shown that in many instances IgG_{2a} is a predominant isotype following immunization with DNA-based vaccines (Ulmer et al., 1996a). It has also been demonstrated that the Th1 cytokine IFN- γ is an important B-cell switch factor for the induction of antigen-specific IgG_{2a} secreting B-cells and that many viral infections in mice result in a predominance of the isotype IgG_{2a} (Snapper and Paul, 1987; Coutelier et al., 1988; Coutelier et al., 1991). Conversely, IgG₁ and particularly IgE antibody production occurs, at least in part, due to the presence of the Th2 cytokine IL-4 (Snapper and Paul, 1987). Of course a variety of other factors, including: mouse strain, dose of antigen, type of pathogen, route of infection/immunization, vaccine formulations, antigen form (soluble or modified) and early induction of IL-6 can all have an impact on the specific character of the developing immune repertoire (Parish and Liew, 1971; Bretscher et al., 1992; Audibert and Lise, 1993; Yang et al., 1993; Aramaki et al., 1995; Guery et al., 1996; Coffman and von der Weid, 1997).

In an effort to further characterize the immune repertoire to a DNA-based vaccine we immunized C3H/HeN (H-2^b) mice with plasmids encoding several deletion mutants of BHV-1 gD. We characterized antigen-specific serum IgG kinetics and duration, as well as predominant IgG isotypes. We also quantified the levels of antigen-specific Th1 and

Th2 cytokines in spleens and draining lymph nodes at various time points post immunization. Our initial data showed that C3H/HeN mice receiving plasmids encoding cell-associated forms (cytosolic or plasma membrane anchored) developed serum isotype profiles that were predominantly IgG_{2a} with an almost exclusive production of the Th1 cytokine IFN- γ following antigen restimulation of splenocytes *in vitro*. We also observed that mice immunized with a plasmid encoding the secreted form of BHV-1 gD displayed a predominance of IgG₁ in serum despite the presence of antigen-specific IFN- γ secreting splenocytes. In an effort to understand the relationship between splenic Th1 profiles and a serum isotype that reflected the influence of IL-4, we determined the antigen-specific levels of IL-4, IFN- γ and IgG isotype secreting B-cells from the iliac lymph node. This node is the primary draining node of the deep tissues of the quadriceps muscle mass. The data showed that draining lymph node cytokine and antigen-specific antibody isotype profiles more accurately reflect the predominant serum isotype of C3H/HeN mice immunized with cell-associated or secreted forms of the BHV-1 gD antigen. The relevance of these observations are discussed in the context of manipulating immune responses by controlling the intra or extracellular location of the antigen when it is first seen by the immune system.

3.2 Materials and Methods

3.2.1 Plasmid Construction

All restriction enzymes and DNA modifying enzymes, as well as markers and

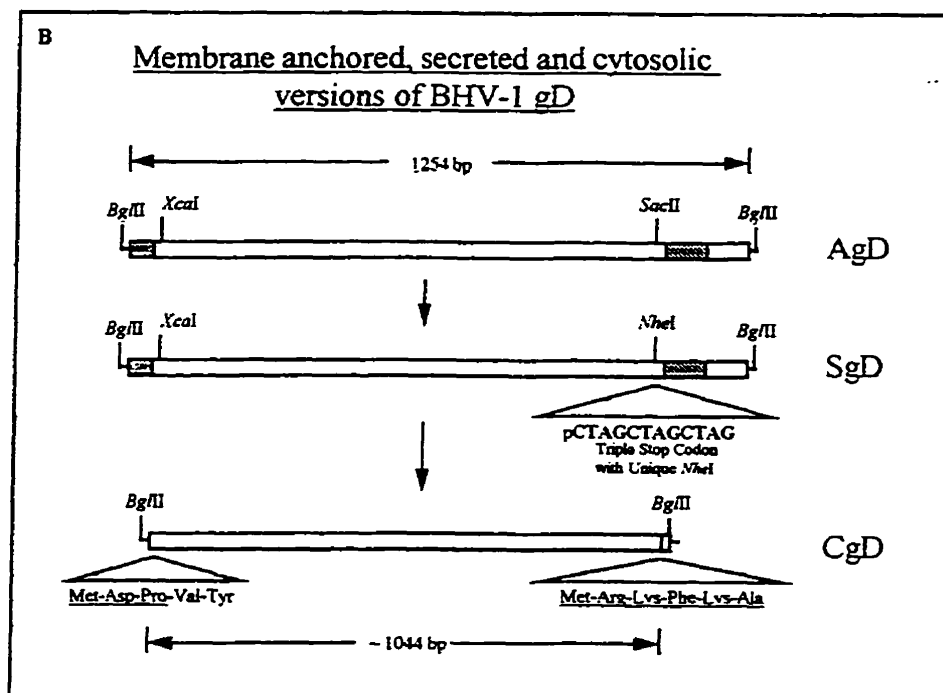
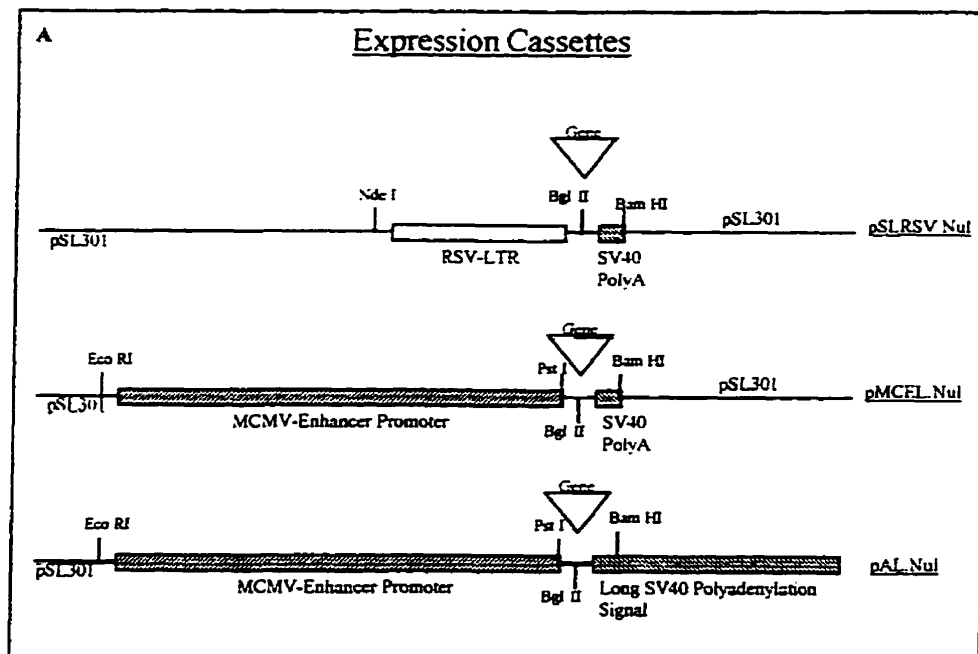
plasmids, were purchased from Pharmacia (Pharmacia Biotech., Quebec) or New England Biolabs (NEB Ltd., Mississauga, Ontario) unless indicated otherwise. Expression cassettes utilizing promoter/enhancer regions from MCMV (Murine Cytomegalovirus) and RSV-LTR (Rous Sarcoma Virus -Long Terminal Repeat) were constructed (see Table 3.1). These regulatory elements were placed in the high copy pSL301[™] (Invitrogen, San Diego, CA) to generate the expression plasmids pMCEL.Nul and PSLRSV.Nul (Fig. 1a). Briefly, pMCEL plasmids were constructed by ablating the existing *Pst*I and *Eco*RI sites within the superlinker region of pSL301 and reintroduction of *Pst*I and *Eco*RI sites, through blunt end linker ligation (*Pst*I linker CCTGCAGG; *Eco*RI linker pGGAATTCC), at the *Eco*RV and *Hpa*I sites, respectively. Vector pSLPNEN-77 was created by ligating the *Eco*RI-*Pst*I fragment containing the MCMV enhancer/promoter from pEnhIE3Mini into *Pst*I-*Eco*RI digested pSLPNEN-77 to create pSLPNEN-77-MCE (Keil et al., 1987; Messerle et al., 1992). The expression cassettes pMCEL.AgD and pMCEL.SgD were generated by ligating the *Bam*HI-*Pst*I fragment from a pSL301 vector containing the SV40 polyadenylation site *Bam*HI-*Bgl*II fragment and the *Bgl*II fragments of the 1300-bp full length BHV-1 glycoprotein D (AgD) and the 1300-bp gene encoding the secreted (SgD) form of gD (Fig. 3.1, A and B). Cleavage of this construct at the *Bam*HI site and shifted *Pst*I site allowed movement of the entire gD sequence and short polyadenylation signal into *Pst*I-*Bam*HI digested pSLPNEN-77-MCE to create pMCEL.AgD and pMCEL.SgD. A pMCEL.Nul plasmid was created by dropping the AgD gene from pMCEL.AgD and religating the plasmids at the *Bgl*II site. The pAL.AgD and pAL.SgD plasmids were generated by ligation of the *Bam*HI-*Ssp*I

Table 3.1 Vectors and plasmids utilized in this study

Plasmids	Relevant characteristics (references)
pSL301 [™]	high copy number cloning plasmid with superlinker region (Invitrogen)
pSLPNEN-77.....	<i>Pst</i> I and <i>Eco</i> RI sites "moved" to <i>Eco</i> RV and <i>Hpa</i> I sites respectively (this study)
pEnhIE3Mini.....	MCMV enhancer and ie3 transcription unit (Keil et al., 1987; Messerle et al., 1992)
pSLPNEN-77-MCE.....	<i>Pst</i> I <i>Eco</i> RI fragment of pEnhIE3Mini inserted into <i>Pst</i> I, <i>Eco</i> RI cut pSLPNEN-77
pMCEL.AgD.....	MCMV promoter + authentic BHV-1 gD (AgD) + SV40 polyA+ signal (this study)
pMCEL.SgD.....	MCMV promoter + secreted BHV-1 gD (SgD) and SV40 polyA+ signal (this study)
pMCEL.Nul.....	MCMV promoter with no gene and SV40 polyA+ signal (this study)
pAL.AgD.....	MCMV promoter + authentic BHV-1 gD and long SV40 polyA+ signal (this study)
pAL.SgD.....	MCMV promoter + secreted BHV-1 gD and long SV40 polyA+ signal (this study)
pSLRSV.AgD.....	RSV promoter with authentic BHV-1 gD and SV40 polyA+ signal (this study)
pSLRSV.SgD.....	RSV promoter with secreted BHV-1 gD and SV40 polyA+ signal (this study)
pSLRSV.CgD.....	RSV promoter with cytosolic BHV-1 gD and SV40 polyA+ signal (this study)
pSLRSV.Nul.....	RSV promoter with no gene and SV40 polyA+ signal (this study)
pRSV1.3.....	RSV promoter with authentic BHV-1 gD on pBR322 backbone (Tikoo et al., 1993)
pRSV1.3*.....	RSV promoter with secreted BHV-1 gD on pBR322 backbone (Tikoo et al., 1993)
pAA505.....	prokaryotic expression cassette derived from pGH433 (Theisen et al., 1992).

fragment of the SV40 polyadenylation signal from pSV2neo[™] (CLONTECH Lab, Inc., Palo Alto, CA) into an end repaired *Bst*EII digest, followed by *Bam*HI digested pMCEL.AgD and pMCEL.SgD (van den Hoff et al., 1993). Plasmids pSLRSV.AgD and pSLRSV.SgD were created by ligating the 947-bp *Nde*I fragment from pRSV1.3 and ligating it into *Nde*I digested pMCEL.AgD and pMCEL.SgD. The *Nde*I fragment from pRSV1.3 contains the RSV-LTR promoter/ enhancer region and a portion of the 5' end of the gD gene. Ligation of a 585-bp *Nde*I-*Bgl*II fragment from pRSV1.3 ligated into *Nde*I-*Bgl*II digested pMCEL.Nul generated plasmid pSLRSV.Nul (Tikoo et al., 1990; Tikoo et al., 1993).

Fig. 3.1 Diagrammatic depiction of expression cassettes. **Fig 3.1 A** depicts three null vectors with RSV LTR or MCMV immediate early enhancer/promoter elements. **Fig. 3.1 B** depicts membrane anchored (AgD), secreted (SgD) and cytosolic (CgD) forms of BHV-1 gD utilized in this research. Expression cassettes all utilized the high copy number pSL301[™] plasmid backbone and enhancer/promoter elements from either RSV-LTR or MCMV. Polyadenylation signal were derived from SV40 as described. All genes encoding full length or truncated versions of BHV-1 gD were inserted at the *Bgl*/II site (▽). The evolution of secreted and cytosolic version of BHV-1 gD are shown (**B**) as are details regarding relevant changes in coding sequences, start codons (CgD) and termination codons. Gene/construct designations are indicated to the immediate right of each diagram.



Creation of an expression cassette expressing a secreted, or truncated, version of BHV-1 gD was described previously (Tikoo et al., 1993). Briefly, plasmid pRSV1.3gDt, which encodes a truncated form of gD was generated by introduction of a unique *Xho*I site at the *Sac*II site immediately adjacent to the transmembrane domain of pRSV1.3 encoding the full length, or authentic, gene for BHV-1 gD. Following restriction digestion and end repair this unique *Xho*I site was blunt-end ligated to a short linker sequence encoding the unique restriction site *Nhe*I and three stop codons (Fig. 3.1, B). Expression of this truncated gene leads to secretion of a glycosylated product of approximately 62-kDa (Tikoo et al., 1993). Plasmid pSLRSV.CgD encodes a cytosolic, or intracellular, version of BHV-1 gD that lacks both the signal sequence and transmembrane domain encoded within authentic, full length, gD (Tikoo et al., 1990). This construct was generated by subcloning the *Xca*I-*Nhe*I subfragment of pSLRSV.SgD into *Xca*I-*Nhe*I digested pSL301. This clone was digested with *Xca*I and *Eco*RV and the gD fragment blunt end ligated into *Sma*I digested pAA505. The prokaryotic expression cassette pAA505 is a derivative of pGH433 with a multiple cloning site consisting of *Nco*I, *Bam*HI, and *Sma*I downstream from a tac promoter (Theisen et al., 1992). Appropriate orientation of the ligation product created a novel start codon contributed by pAA505 followed by Asp and Pro prior to in-frame commencement of the gD amino acid sequence Tyr Val Asp Proimmediately downstream from the signal peptide sequence (Fig. 3.1, B). Clone pAACgD was digested with *Dde*I and *Eco*47III, end repaired with the Klenow fragment of *E. coli* DNA polymerase I and blunt end ligated to a *Bgl*II linker containing in-frame stop codons (GTAGCTAGATCTG). This ligation product was then

digested with *Bgl*II , purified with Geneclean Kit (BIO 101, Inc., Vista, CA), and ligated into *Bgl*II digested pSLRSV.Nul. This construct displays 6 additional amino acids (Met-Arg-Lys-Phe-Lys-Ala), contributed by the *Nhe*I-*Eco*RV fragment of pSL301, at the carboxy terminus.

3.2.2 Bacterial Hosts, Mammalian Cell Lines and Tissue Culture Reagents.

JM105 (New England Biolabs, Inc.), HB101 (Invitrogen), and DH5 α (CLONTECH Laboratories, Inc.) were the bacterial strains used to amplify plasmid DNA. Murine L929 connective tissue cell line was received from American Type Culture Collection (ATCC, Rockville, MD.), (NCTC clone 929). Madin Darby Bovine Kidney (MDBK) cell line was obtained from ATCC (# CCL 22). C3H/HeNCrIBR and C57BL/6 mice were purchased from Charles River (Charles River, St. Constante, Quebec). All media and media supplements were purchased from GIBCO/BRL (GIBCO/BRL, Burlington, Ontario) or Sigma (Sigma Chemical Co., St. Louis, MO), unless otherwise indicated. All tissue culture plasticware was purchased from Corning (Corning Inc., Corning, N.Y.) or Costar (Costar/Nucleopore Canada Inc., Toronto, Ontario), unless indicated otherwise.

3.2.3 Stable Transfections

Plasmids encoding secreted or cytosolic versions of BHV-1 gD were mixed with a pSV2neo (CLONTECH) encoding G418 (Geneticin[™]; GIBCO/BRL) resistance at a ratio of 8 μ g :2 μ g, respectively, and used to transfect L929 cells. Lipofectin[™]

(GIBCO/BRL) mediated transfection was carried out using the following protocol: DNA was incubated with Lipofectin[™] for 15 minutes in Optimem (GIBCO/BRL) media, preconfluent cell monolayers were washed several times with warmed Optimem and after the final wash the DNA:Lipofectin mixture was added. The DNA:Lipid mixture was removed after 6 hours and the cells incubated overnight in MEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co.), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma Chemical Co.), 2 mM L-glutamine (GIBCO/BRL) 1mM sodium pyruvate (GIBCO/BRL) . Monolayers were split (following trypsinization) to ten 100 mm tissue culture dishes and maintained in complete MEM with 400 µg/mL G418 for 10-14 days. Media was changed every 3 days during this time. Single G418 resistant colonies were harvested using silicon greased cloning cylinders (Bellco Glass Inc., Vineland, N.J.) and trypsinization. Harvested colonies were transferred to individual wells of either 96 or 48 well tissue culture plates. Individual G418 resistant clones were expanded to 24 well plates and the culture fluids were harvested for detection of secreted BHV-1 gD. Monolayers were harvested for SDS-PAGE using a modified NP-40 lysis buffer protocol (Harlow and Lane, 1988). Putative gD secreting stable transfectants were harvested into freezing media (90% FBS and 10% dimethylsulfoxide, sterile filtered) and frozen overnight at -70 °C. Frozen stable transfectants were then transferred into liquid nitrogen until needed.

Immunoprecipitation of SgD involved mixing of 1.0 mL of fresh or thawed (following storage at -70 °C) media, harvested from confluent G418 resistant clones, with 200 µL of 5x binding buffer (750 mM NaCl, 200 mM Tris-HCl, pH 7.6; 10 mM EDTA, pH 8.0;

0.5% NP-40) containing 5 mM phenylmethylsulfonyl fluoride (PMSF) and 1.0 μ L of an anti-gD monoclonal antibody pool (mAbs \rightarrow 9D6, 3D9, 10C2, 2C8, PB136, 3E7, 3C1 and 4C1) at a dilution of 1.5:10 (in 1x binding buffer) (Hughes et al., 1988; van Drunen Littel-van den Hurk et al., 1990). Binding was allowed to proceed for 3 hours at 4 °C, with mixing, on an Adams nutator (Model 1105, Clay Adams, Division of Becton Dickinson and Co., Parsippany, N.J.). Protein G Sepharose (Pharmacia) in a modified RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.5% sodium dodecylsulfate, 10 mM EDTA) was mixed with the samples and allowed to bind for 3-4 hours. Samples were precipitated by spinning for 30 seconds in an Eppendorf Centrifuge 5414 (Brinkman Instruments, Rexdale, Ontario), (16,000 x g). Pellets were washed two times with 1x binding buffer and then solubilized in a nonreducing (no β -mercaptoethanol) SDS-PAGE loading buffer. Samples were loaded onto a 10 % SDS-PAGE minigel (Bio-Rad Lab. Ltd., Mississauga, Ontario) and electrophoresed at 120 volts for 60 minutes. Gels were electroblotted onto nitrocellulose (BIO-RAD) using the recommended blotting procedure (Bio-Rad). Nitrocellulose blots were blocked for 1-2 hours with 3% gelatin (Bio-Rad) in Tris-buffered saline with 0.05% Tween-20 (Sigma Chemical Co.) and then incubated for 1-2 hours with a 1:1000 dilution of the anti-gD monoclonal pool in Tris-buffered saline with Tween 20 (TBST) with 1% BSA. Development involved a 90 minute incubation with a biotinylated goat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA) at 1:1000 dilution followed by a 60 minute incubation with a 1:1000 dilution of Streptavidin-alkaline phosphatase conjugate (GIBCO/BRL). Development was carried out using 5-

bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) substrates (Bio-Rad). The development reaction was stopped by extensive washing in tapwater.

3.2.4 Transient Transfection of COS-7 cells with pSLRSV.AgD, pSLRSV.SgD and pSLRSV.CgD.

COS-7 cells at 70-80% confluency in six-well tissue culture plates, were transfected with 5 µg of plasmid DNA for 5 hours in serum free Optimem™. Lipofectamine™ (GIBCO-BRL) was utilized to facilitate transfection at a ratio of 5µg Lipofectamine:1µg plasmid DNA. DNA was purified with Qiagen columns (Qiagen Inc., Santa Clarita, CA) and combined with Lipofectamine as described in the protocol insert. Optimem™ containing DNA:Lipid complexes was removed from cells after 5 hours and complete media containing glucose (DMEM) and 15 % FBS was added to the cells. After 12 hours transfected cell monolayers were washed with warm phosphate buffered saline (PBS-0.137 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M NaH₂PO₄) and 1.5 mL of methionine free MEM containing 2% dialysed FBS and 50 µCi/mL of ³⁵S-labelled L-Methionine (Tran³⁵S-Label™, ICN Pharmaceuticals, Inc., St. Laurent, Quebec) was added to each well. Cells were incubated for 24 hours and harvested for immunoprecipitation. Media was collected and centrifuged (16,000 x g) in 1.5 mL eppendorf tubes for 5 minutes to remove debris. Supernatants were aspirated to clean labelled tubes and either processed immediately or stored at -20 °C until needed. Immunoprecipitation of gD from supernatants was carried out by mixing 800 µL of media with 200 µL of 5x binding buffer containing the protease inhibitors: Aprotinin, Leupeptin, Antipain, PMSF (40

µg/mL, 5 µg/mL, 5 µg/mL and 200 µg/mL, respectively), and 1 µL of a 1:10 dilution of an anti-gD mAb pool. Samples were incubated, at 4 °C, for 3 hours on a nutator.

Precipitates were collected by centrifugation after incubation with 100 ng Sepharose-G equilibrated in RIPA buffer. Detection of cell membrane associated gD was carried out by extensive washing of transfected cell monolayers, with warm PBS, followed by a 37 °C incubation of monolayers in 500 µL PBS containing 6.0 µL of a 1:10 dilution of an anti-gD mAb pool. After a 60 minute incubation, the monolayer was washed extensively and lysed on ice for 10 minutes in 500 µL Dissociation buffer (0.5 % NP-40, 0.5% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.4) containing the protease inhibitors: Aprotinin, Leupeptin, Antipain and PMSF, at final concentrations of , 8.0, 1.0, 1.0 and 40.0 µgs/mL, respectively. After incubation, solubilized monolayers were pipetted up and down several times and collected into a 1.5 mL Eppendorf tube. Samples were centrifuged (16,000 x g) for 2 minutes in an Eppendorf centrifuge and supernates transferred to a clean 1.5 mL Eppendorf tube. Samples were mixed with 200 µL of RIPA buffer containing protease inhibitors and 120 µL Sepharose-G (100 ng) equilibrated in RIPA buffer. Samples were incubated for 3 hours, at 4 °C, on an Adams nutator prior to harvest.

Cell lysates were prepared as described for detection of membrane associated gD except that the pre-incubation with an anti-gD mAb pool was eliminated. Following a 2 minute centrifugation (16,000 x g), cleared supernatants were transferred to new 1.5 mL Eppendorf tubes. Aliquots (200 µL) of these cell lysates were immunoprecipitated by mixing with 800 µL of RIPA buffer containing protease inhibitors and 1 µL of a 1:10

dilution of anti-gD mAb pool. These samples were incubated for 3 hours, at 4 °C, on a nutator. After this primary incubation, immunoprecipitates were harvested by incubating for 3 hours with 100 ng Sepharose-G and centrifugation (16,000 x g) for 30 seconds in an Eppendorf microfuge.

All immunoprecipitated pellets were washed 2 times in 500 µL cold RIPA buffer containing protease inhibitors. Following the final wash, pellets were resuspended in 40 µL of a 1.5 x reducing SDS-PAGE loading buffer. Samples were mixed, boiled for 3 minutes and chilled on ice for 5 minutes prior to loading on a 12 x 16 cm SDS-PAGE gel (4% stacking and 10% separating). Electrophoresis was carried out for 4 hours at 180 volts. Gels were fixed (40% methanol, 10 % acetic acid) for 15 minutes and equilibrated for 30 minutes in a fluorographic agent (Amplify™, Amersham, Oakville, Ontario). Gels were dried, under vacuum, at 80 °C for 2 hours and exposed to preflashed Kodak X-OMAT™ AR film for 12-24 hours.

3.2.5 Mouse Injections and Identification of Draining Lymph Nodes

Inbred 7 week old, female C3H/HeNCrlBR mice were injected with column purified (QIAGEN Inc.) plasmid DNA dissolved in normal saline. All injections of DNA were i.m. while injections of purified antigen (authentic or recombinant truncated gD) formulated in either VSA3 (Biostar, Saskatoon, Saskatchewan) or alum (Alhydrogel™, Cedarlane Laboratories Ltd., Hornby, Ontario) were subcutaneous (s.q.). Mice in experiment I (Fig. 3.4) received injections of 100 µg plasmid DNA (2.0 µg/ µL in normal saline) in the left quadriceps only. All other mice received injections of 50 µg of

plasmid DNA in the left and right quadriceps muscle mass using DNA at a concentration of 1.0 $\mu\text{g}/\mu\text{L}$. All mice were boosted at 14 days. Nonlethal tail bleeds were carried out every 2 weeks until the experiments were terminated. Identification of the lymph nodes draining the quadriceps muscle mass was carried out by i.m. injection of 7 week old C3H/HeN mice with either a 2% Evans blue dye (in normal saline) or column purified plasmids pSLRSV.AgD or pSLRSV.Nul. Mice receiving Evans Blue dye were euthanized with a Halothane[™] (MTC Pharmaceutical, Cambridge, Ontario) overdose between 5 and 20 minutes after injection. Colour photographs of dissected dye injected mice were taken using a Nikon F3 camera with a macro lens and Ektachrome 60 color film. DNA injected mice were euthanized at either 20 minutes or 20 hours post-injection and iliac or inguinal lymph nodes flash frozen in liquid nitrogen. Flash frozen lymph nodes were stored at -70 °C, in labelled 1.5 mL microfuge tubes, until isolation of cellular DNA and amplification of a portion of BHV-1 gD by polymerase chain reaction (PCR) could be carried out. Isolation of DNA for PCR was carried out using a Proteinase K digestion protocol (Wright and Manos, 1990). PCR was carried out using the following primers: Sense primer-TTCTCGAAACTCGGCGCGGCT and antisense primer-TTCGAGGCTCGGC CAGCCTTC . These primers generate a 435 bp product that commences at base 531 downstream from the start codon (Tikoo et al., 1990). The PCR reaction for detection of BHV-1 has been described previously (Vicek, 1993). Briefly, detection of the BHV-1 gD sequence, using PCR, was carried out in a final volume of 100 μL , using *Taq* polymerase, and included: 1.0 μL of DNA prepared from lymph nodes, 5 mM dNTPs, 50 pmol oligonucleotide primers and glycerol (final concentration

of 10 %). Following 8 minutes of denaturation at 95 °C (under a mineral oil overlay), 5 minutes of quick chilling on ice, the sample was cycled on a thermocycler using the following parameters: denaturation at 95 °C for 70 seconds., annealing at 56 °C for 60 seconds, and extension at 73 °C for 50 seconds. Polymerase chain reaction products were assessed by electrophoresis on 1.2 % agarose gel in the presence of ethidium bromide.

3.2.6 Splenic and Lymph Node Cell Harvests

Mouse splenocytes were prepared by mechanically grinding spleens through a sterile nylon mesh into a sterile Petri dish containing 10-20 mL of RPMI 1640 (Sigma Chemical Co.). This material was then pipetted into sterile 15 or 50 mL tubes and allowed to settle on ice for 10 minutes. Debris that settled to the bottom was aspirated with a sterile Pasteur pipette and discarded. Tubes were centrifuged at 250 x g, in a Beckman GPR centrifuge, for 10 minutes (at 4 °C). Red blood cells were lysed by resuspension of cell pellets in 1.0 mL/spleen of ammonium chloride lysis buffer (0.14 M NH_4Cl and 0.017 M Tris-HCl, pH 7.2) for 60 seconds. Lysis was terminated by the addition of 14 mL of RPMI 1640 at ambient temperature. Samples were pelleted by centrifugation at 250 x g, and resuspended in RPMI 1640 media. Splenocyte numbers were assessed by staining with trypan blue (GIBCO/BRL) and counting with the aid of a haemocytometer. Splenocytes were centrifuged (250 x g) and resuspended at a final concentration of 1×10^7 cells/mL in RPMI 1640 supplemented with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma Chemical Co.), 2 mM L-glutamine (GIBCO/BRL), 1 mM sodium pyruvate (GIBCO/BRL), 10 mM HEPES (GIBCO/BRL)

and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.), (complete RPMI-1640).

A modification of the method for harvesting Peyer's patch lymphocytes was utilized to prepare single cell suspensions from lymph nodes (Spalding et al., 1983). Briefly, lymph nodes were collected into 15 mL tubes containing sterile chilled phosphate buffered saline without calcium or magnesium (PBSA) (GIBCO/BRL). Pooled nodes were decanted into a sterile Petri dish and excess PBSA discarded. Nodes were diced with a sterile scalpel in a small volume of CMF solution {0.1 x Hanks Balanced Salt Solution (HBSS), Ca^{2+} Mg^{2+} free; 10 mM Hepes, pH 7.2; 25 mM NaHCO_3 , pH 7.2; and 2% FBS} and then mixed with 10 mL digestion buffer (1 x HBSS, 10% FBS, 15 mM HEPES, pH 7.2) containing 150 Units/mL of Collagenase (CLS 1, Worthington Biochemical Corporation), and 0.015 mg/mL DNase I (Pharmacia Biotech. Inc.). Diced nodes in digestion buffer were transferred to silanized 25 -50 mL Erhlenmeyer flasks, containing a sterile Teflon coated stir bar, and incubated at 37 °C, with stirring, for 90 minutes. Samples were harvested into sterile 15 mL plastic tubes and allowed to sit for 10 minutes. Large pieces of undigested material were aspirated back into the Erhlenmeyer flask and incubated an additional 30-60 minutes with 5.0 ml of digestion buffer. This material was combined with the initial harvest and cells were pelleted by centrifugation (250 x g). Digestion buffer was decanted and the cells were resuspended in phosphate buffered saline without Ca^{++} (PBSA), (GIBCO/BRL). Cells were counted using a haemocytometer and centrifuged (250 x g) one final time. Cells were resuspended to a final concentration of 1×10^7 cells/mL.

A modified protocol for splenocytes included an additional 60 minute

digestion of pooled splenocytes with collagenase (150 Units/mL of digestion buffer) and DNase I (0.015 mg/ml of digestion buffer). These digestions were only carried out in experiments that assessed antigen-specific responses from cells prepared from lymph nodes. These digestions were carried out prior to cell counts and resuspension.

3.2.7 Total IgG and IgG Isotype ELISA

Immunolon 2 microtitre plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with purified recombinant truncated gD (Biostar Inc.) at a concentration of 0.050 µg/well. Coating antigen dilutions were done in ELISA Coating buffer (.012 M Na₂CO₃, .038 M NaHCO₃, pH 9.6) and coating was allowed to proceed overnight at 4 °C. Plates were washed 5 times in phosphate buffered saline (.137 M NaCl, .003 M KCl, .008 M Na₂HPO₄, .001 M NaH₂PO₄) with 0.05% Tween-20 (PBST) prior to addition of fourfold dilutions of mouse sera prepared in PBST with 0.5% gelatin (PBST-g), (Bio-Rad Laboratories Ltd., Mississauga, Ontario). After a 2 hour incubation, plates were washed in PBST and an affinity purified biotinylated goat antimouse Ig (Zymed Laboratories Inc., San Francisco, CA) diluted to 1/5,000 in PBST-g was added to each plate. After an incubation of 60 minutes, plates were washed extensively and Streptavidin-alkaline phosphatase (Gibco, Life Technologies, Burlington, Ontario) diluted to 1/2000, in PBST-g, was added to each plate. Following a 60 minute incubation, plates were washed six times in PBST. Prior to addition of substrate the plates were washed two additional times in PBS. Development of plates involved the addition of .01 M p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in substrate buffer (0.104 M Diethanolamine-

Sigma Chemical Co., 0.5 mM MgCL₂). Absorbances were read on a Model 3550 Microplate Reader (Bio-Rad Laboratories Ltd.) at 30 minutes and again at 60 minutes at 405 nm with a reference wavelength of 490 nm. Antibody isotyping ELISAs were carried out in a similar fashion except that biotinylated goat antimurine IgG₁ , IgG_{2a} , IgG_{2b} , IgG₃ , IgM (Caltag Laboratories, San Francisco, CA) were used at a dilution of 1/8,000 except for IgM (1/2,000). Incubation times with these antibodies was 60 minutes. Addition of Streptavidin-alkaline phosphatase and development of plates was as described for total IgG ELISA.

3.2.8 ELISPOTs.

A cytokine specific ELISPOT assay was used as described previously (Czerkinsky et al., 1988; Baca-Estrada et al., 1996). Briefly, single cell suspensions isolated from spleens, lymph nodes or bone marrow were stimulated *in-vitro* by incubating in complete RPMI 1640 at 37 °C and 5% CO₂ for 20 hours in the presence of recombinant authentic gD (0.4 µg/mL). After antigen stimulation, cells were washed twice in complete media and diluted to a concentration of 1 x 10⁷ cells/mL of complete RPMI-1640. Nitrocellulose plates (FILTApate™, 0.45 µm; Polyfiltronics, Inc., Rockland, MA, USA) were prepared by coating overnight at 4 °C with 2.5 µg/mL of purified antimurine interleukin (IL)-4 (Cat. No. 11B11) or 5 µg/mL of purified antimurine interferon (IFN)-γ (Cat No. R4-6A2) (Pharmingen, San Diego, CA, USA) diluted in carbonate/bicarbonate buffer (45.3 mM NaHCO₃, 18.2 mM Na₂CO₃, pH 9.6). After coating, unbound antibody was washed from the wells thrice with sterile PBST followed by 3 washes with sterile

PBS. Wells were blocked for 2 hours at 37 °C with complete RPMI 1640. Blocking media was decanted and 100 µL of antigen stimulated cell suspensions were added to triplicate wells. After a 20 hour incubation, at 37 °C and 5% CO₂, plates were washed 2 times in tapwater and 5 times in PBST to remove cells and nonspecifically bound cytokine. One hundred µL of biotinylated antimurine IL-4 (Cat. No. BVD6-24G2) and IFN-γ (Cat. No. XMG1.2) (Pharmingen Canada Inc.) monoclonal antibodies (3 µg/mL of each in 1% FBS/PBS) were added to appropriate wells and incubated for 4-6 hours at ambient temperature on an oscillating mixer. Plates were washed 6 times in PBST and 100 µL of a 1/1000 dilution of Streptavidin alkaline phosphatase (Bio/Can Scientific, Mississauga, Ontario, Canada) in 1% FBS/PBS was added to each well. After a 2 hour incubation, at ambient temperature, the plates were washed twice in PBS. Plates were developed by the addition of 50 µL of the substrates, 5-bromo-4-3-indolyl phosphate (BCIP), and nitroblue tetrazolium (NBT), (Moss Inc., Pasadena, MD). Plates were allowed to develop at ambient temperature for 10-30 minutes, after which reactions were stopped by extensive washing in distilled water. After air drying, spots were counted under a dissecting microscope. B-cell ELISPOT involved the use of nitrocellulose plates (Millipore Milliscreen-HA, Millipore, Bedford, MA) that were coated overnight at 4 °C with purified recombinant truncated gD at 5.0 µg/mL in carbonate/bicarbonate buffer (pH 9.6). Coated plates were washed three times in sterile PBST followed by 3 washes in sterile PBS. Nonspecific binding sites were then blocked with complete RPMI 1640 at 37 °C for 1-2 hours. Fresh, unstimulated cells from spleen or lymph nodes, prepared as

described above, were added in a volume of 100 μL (1×10^7 cells/mL) to each well. Uncoated but blocked control wells, for nonspecific binding of IgG, also received cells from each test sample in triplicate. Plates were incubated at 37 °C with 5% CO_2 for 10-12 hours. After the incubation period, all cells were washed out of the wells by 2 washes with tapwater followed by 6 washes with PBST. Biotinylated antimurine IgG₁ or IgG_{2a} antibodies (1/8,000 dilution) were added in a 100 μL volume of PBST (with 1% FBS) and incubated with mixing for 3-4 hours at ambient temperature. After washing in PBST six times, streptavidin-alkaline phosphatase (1/1,000 dilution in PBS-T with 1% FBS) was added to each well (100 μL). After a 60 minute incubation, plates were washed six times in PBST and 2 times in PBS. Plates were developed as described for the cytokine ELISPOT.

3.2.9 Serum Neutralization Assays

Sera from mice immunized with DNA-based, or conventional vaccines were assayed for virus neutralizing capacity by a BHV-1 plaque reduction assay as described previously (Babiuk et al., 1975). Briefly, 110 μL of 2- or 4-fold dilutions of heat inactivated sera (in RPMI 1640 containing 2% FBS and 1:40 dilution of guinea pig complement; GIBCO/BRL) were mixed with 110 μL of live BHV-1 for 60 minutes. Following incubation, 100 μL of each dilution were placed on confluent MDBK cells, in duplicate, in a 96 well flat bottomed culture plate (Nunc Inc., Naperville, IL). Plates were incubated for 48 hours and plaques were visualized by decanting the media overlay and

staining cells with (1% Crystal violet in 70% ethanol). Neutralizing titers are expressed as the reciprocal of the highest dilution of antibody that caused 50% reduction of plaques relative to the virus control wells.

3.2.10 Statistical Analysis

Data was entered into a database in the statistical analysis program Instat GraphPad™. Differences in antibody titers among vaccine groups were investigated using the nonparametric Mann-Whitney *U* test.

3.3 Results

3.3.1 *In vitro* transfection of COS-7 and L929 cells.

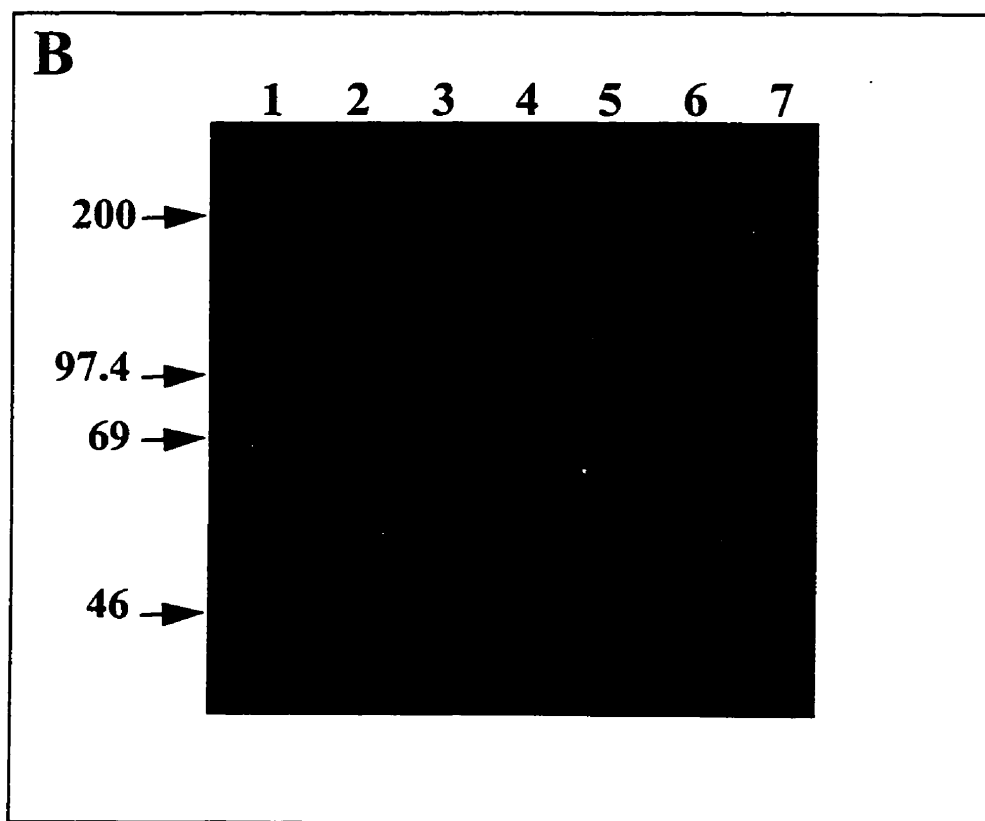
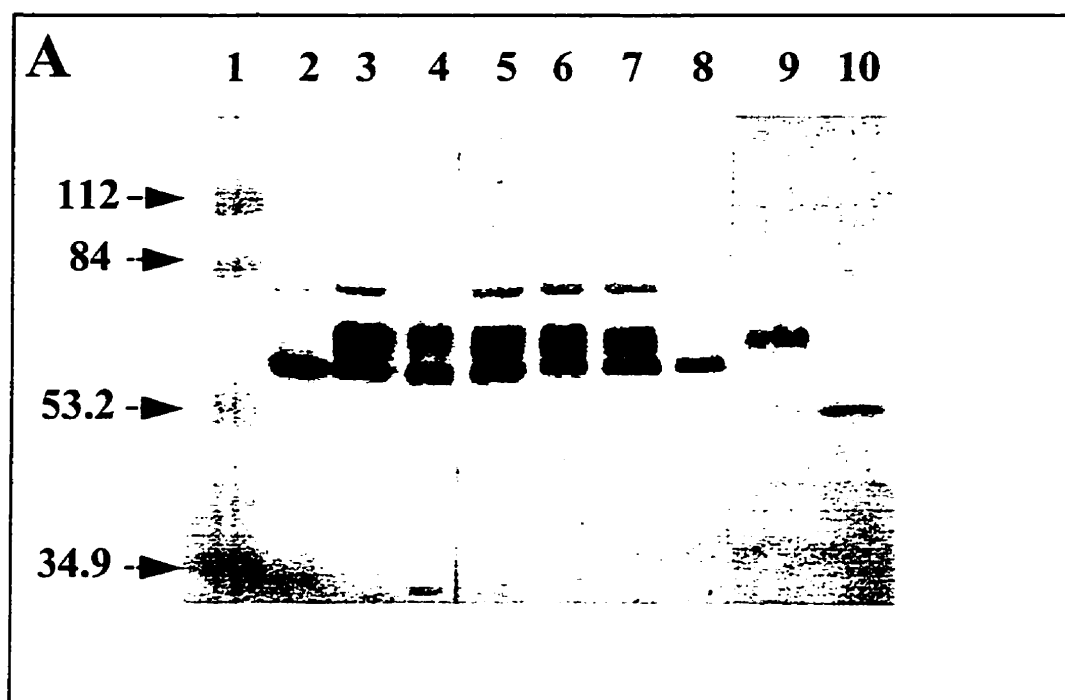
Transient and stable transfection of COS-7 and L929 cells with expression plasmids demonstrate function and cellular compartmentalization of BHV-1 gD deletion mutants. Table 3.1 and Fig.3.1 depict expression cassette nomenclature and evolution of expression cassettes utilized in this study. Although *in vitro* expression of the authentic and secreted forms of BHV-1 gD has been described previously (Tikoo, 1990; Tikoo, 1993), we felt that subcloning of genes and novel regulatory elements to pSL301™ justified further assessment. We also needed to ensure that the cytosolic (CgD), authentic (AgD), and secreted (SgD) forms of BHV-1 gD occurred within the appropriate, and predicted, cell compartment. Fig. 3.2 A clearly demonstrates that RSV-LTR and MCMV immediate early promoter driven secretion of the truncated version of BHV-1 gD occurs

effectively. It is difficult to accurately assess the level of expression from this immunoprecipitation data despite the fact that this information was generated from stable transfected subclones. These stable transfected lines grew with distinct kinetics and media requirements which made expression strength comparisons between clones difficult. These cell lines were also prone to exhibiting an inexplicable loss of expression. However, radioimmunoprecipitation of 4 of the 5 subcloned lines (the specific subclone stable transfected with pMCEL.SgD lost the ability to express gD) suggested that the pAL.SgD construct outperformed the RSV-LTR driven constructs *in vitro* (Fig. 3.2, B) It is difficult to attribute this apparent enhanced expression to either, the presence of the MCMV immediate early enhancer/promoter element, or the longer SV40 polyadenylation. However, *in vivo* evidence suggests the presence of the longer polyadenylation signal contributes to the expression strength of this cassette (refer to serum IgG titer levels in mice immunized with pMCEL constructs versus pAL constructs in Fig. 3.4, and Table 3.2). Despite early, *in vitro* evidence that pAL constructs outperformed the RSV-LTR constructs, the pAL plasmids were difficult to propagate in several bacterial strains. These plasmids would only replicate in HB101 *E. coli* and yields were typically lower than observed for plasmids containing the RSV-LTR elements and grown in DH-5 α bacterial. For these reasons, and because *in vivo* data suggested there was little advantage to be gained from using the pAL constructs, all subsequent experiments involved the use of pSLRSV constructs. COS-7 cells transiently transfected with plasmids: pSLRSV.AgD, pSLRSV.SgD and pSLRSV.CgD clearly show that these constructs express gD predominantly at the plasma membrane (pSLRSV.AgD),

Fig. 3.2 *In vitro* expression from plasmids encoding a secreted form of BHV-1 gD. Western blot (3.2 A) and radioimmunoprecipitate (3.2 B) analysis of media from L929 cells stably transfected with expression cassette encoding the secreted form of BHV-1 gD (SgD). Cultures at ~80% confluency, in six well plates received 2.0 mL of media and were incubated for ~30 hours. Unlabelled and radiolabelled secreted gD was immunoprecipitated from media (1.0 mL) with a monoclonal pool and Sepharose G. Immunoprecipitated proteins were mixed with a nonreducing loading buffer and separated on a SDS-PAGE minigel (3.2 A) or a 12 x 16 cm gel (3.2 B). Western blot analysis (3.2 A) involved: transfer to nitrocellulose and detection of gD with anti-gD mAb pool followed by biotinylated Goat anti-mouse IgG and a Streptavidin alkaline phosphatase Figure conjugate. Colour development was carried out using BCIP and NBT.

Figure 3.2 A depicts the following: Lane: 1, Prestained low molecular weight markers (Biorad[™]); 2, 1.0 uL of 1 in 10 uL dilution of mAb pool; 3, immunoprecipitate from L929 transfected with pAL.SgD; 4, immunoprecipitate from L929 transfected with pSLRSV.SgD; 5, immunoprecipitate from L929 transfected with pMCES.SgD (shortened version of MCMV enhancer/promoter element not described in methods); 6, immunoprecipitate from L929 transfected with pRSV.gIVt1.3; 7, immunoprecipitate from L929 transfected with pMCEL.SgD; 8, immunoprecipitate from L929 cells transfected with pSLRSV.Nul; 9, 100 ng affinity column purified recombinant secreted (truncated) gD; 10, 100 ng of *E. coli* aggregate preparation of cytosolic gD expressed from pAA505, respectively.

Figure 3.2 B depicts ³⁵S labelled gD from 4 of 5 stably transfected L929 cell lines was immunoprecipitated as described above and resolved on a 10% SDS-PAGE gel (3.2 B). Lanes: 1, lysate from BHV-1 infected MDBK cells; 2, secreted gD from L929 cells infected with a recombinant Vaccinia expressing a secreted form of gD; 3, pAL.SgD transfected cell line; 4, pSLRSV.SgD transfected cell line; 5, pMCES.SgD transfected cell line; 6, pRSV1.3*; 7, pSLRSV.Nul transfected cell line. The molecular weights of the prestained markers are indicated to the left.



extracellularly (pSLRSV.SgD), or in the cytoplasm (pSLRSV.CgD) (Fig. 3.3). Molecular weights were as predicted based on cloned sequence data and previous work.

3.3.2 Serum IgG kinetics in DNA-immunized mice.

Immunization of C3H/HeN mice with a plasmid encoding an intracellular form of BHV-1 gD display antibody kinetics that differ from mice receiving cell surface or secreted forms of BHV-1 gD. Fig. 3.4 shows that mice from all groups immunized with the plasmids encoding the authentic (pSLRSV.AgD) or secreted (pSLRSV.SgD) versions of BHV-1 seroconvert as early as 2 weeks following the initial immunization.

Interestingly, mice immunized with plasmid encoding the intracellular version of gD (pSLRSV.CgD) showed a 2 to 4 week lag in serum titer development as compared to mice immunized with constructs encoding membrane anchored antigen (AgD constructs), or secreted (SgD constructs), antigen. Not all mice immunized with plasmid DNA, with the exception of the group immunized with pAL.SgD, seroconverted in this experiment. Early, mid, and late total IgG serum titer kinetics were similar for mice immunized with plasmids encoding the authentic or secreted versions of gD. Mean serum titers for mice receiving the cytosolic version of gD (pSLRSV.CgD) tended to be lower than mice immunized with other plasmid constructs. Table 3.2 shows that mean serum titers for all DNA groups were higher than those seen in mice immunized with purified viral gD or recombinant truncated gD (formulated in Alum or VSA3). At 12 weeks, mice immunized with either pAL or pSLRSV constructs displayed mean titers greater than those seen in mice immunized with conventional vaccine formulations. Fig.

Fig. 3.3 Autoradiograph of immunoprecipitated forms of membrane anchored, secreted and cytosolic BHV-1 gD. Preconfluent COS-7 cells transiently transfected with pSLRSV.AgD, pSLRSV.SgD, pSLRSV.CgD or pSLRSV.Nul were grown for 48 hours in the presence of ^{35}S labelled methionine and cysteine. Radioactively labelled gD was immunoprecipitated from media and/or cell lysates using an anti-gD mAb pool. SDS-polyacrylamide gel electrophoresis of precipitates demonstrates calculated m.w. of mutated gD and cellular localization as predicted. Immunoprecipitates of media collected from COS-7 transfected with: Lane 1, pSLRSV.AgD; 2, pSLRSV.SgD; 3, pSLRSV.CgD; and 4, pSLRSV.Nul, respectively. Immunoprecipitates of lysates of COS-7 cells transfected with: Lane 5, pSLRSV.AgD; 6, pSLRSV.SgD; 7, pSLRSV.CgD, and 8, pSLRSV.Nul, respectively. Immunoprecipitates of plasma membrane associated gD are shown in Lanes 9, pSLRSV.AgD; 10, pSLRSV.SgD; 11, pSLRSV.CgD, and 12, pSLRSV.Nul, respectively. Molecular weight markers are indicated on the left.

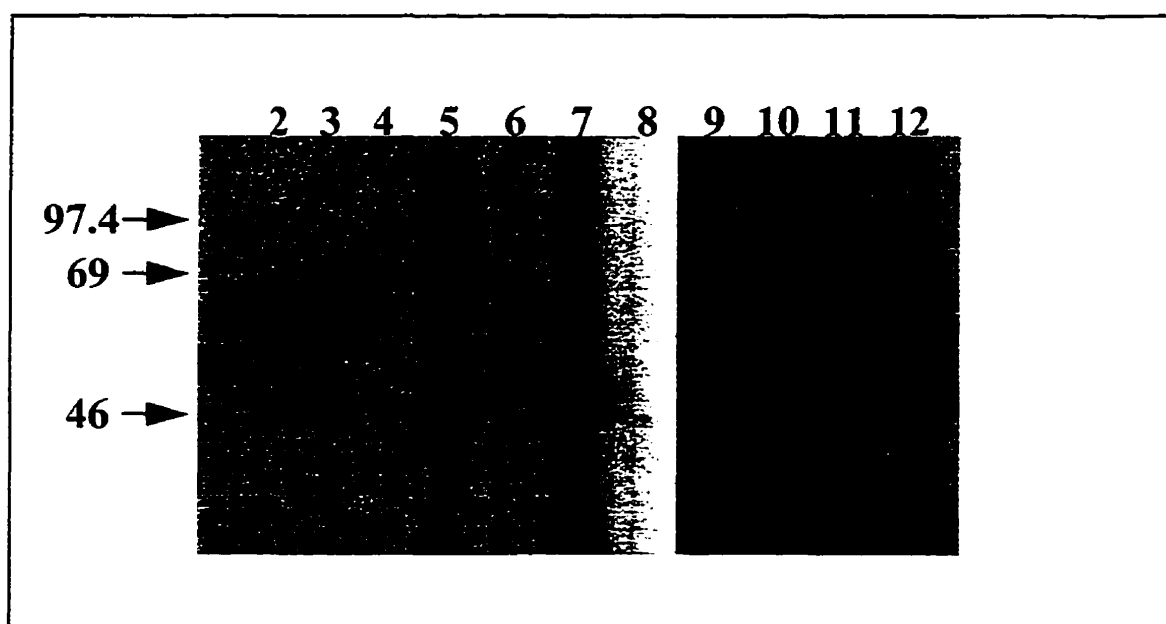


Fig. 3.4 Kinetics of serum anti-BHV-1 gD antibodies in C3H/HeN mice immunized with DNA or conventional vaccines. Serum ELISA titers were determined by using the extrapolation function (Microsoft[™] Excel) based on endpoint dilutions and using preimmune sera means (plus 3 standard deviations) as cutoffs. Endpoint titers are expressed as $1/\log_{10}$. Experimental groups contained 5 (DNA-based vaccines) or 3 mice (Alum or VSA formulations). Bleeds from each mouse were determined individually for each time point except at 2 weeks when bleeds for each group were pooled. Only 23 week titers from mice that were seropositive at week 14 were determined. All DNA-based vaccines were administered intramuscularly in the left quadriceps muscle mass (superficial and/or deep muscles of the thigh), with doses of 100 μg (2 $\mu\text{g}/\mu\text{L}$ in normal saline) at days 0 and 14. Affinity purified, full length, BHV-1 gD (AgD), and recombinant truncated gD (tgD), were given at doses of 200 $\eta\text{g}/\text{mouse}$ subcutaneously following formulation with VSA or Alum. Vaccine designations are indicated in the top centre of each graph.

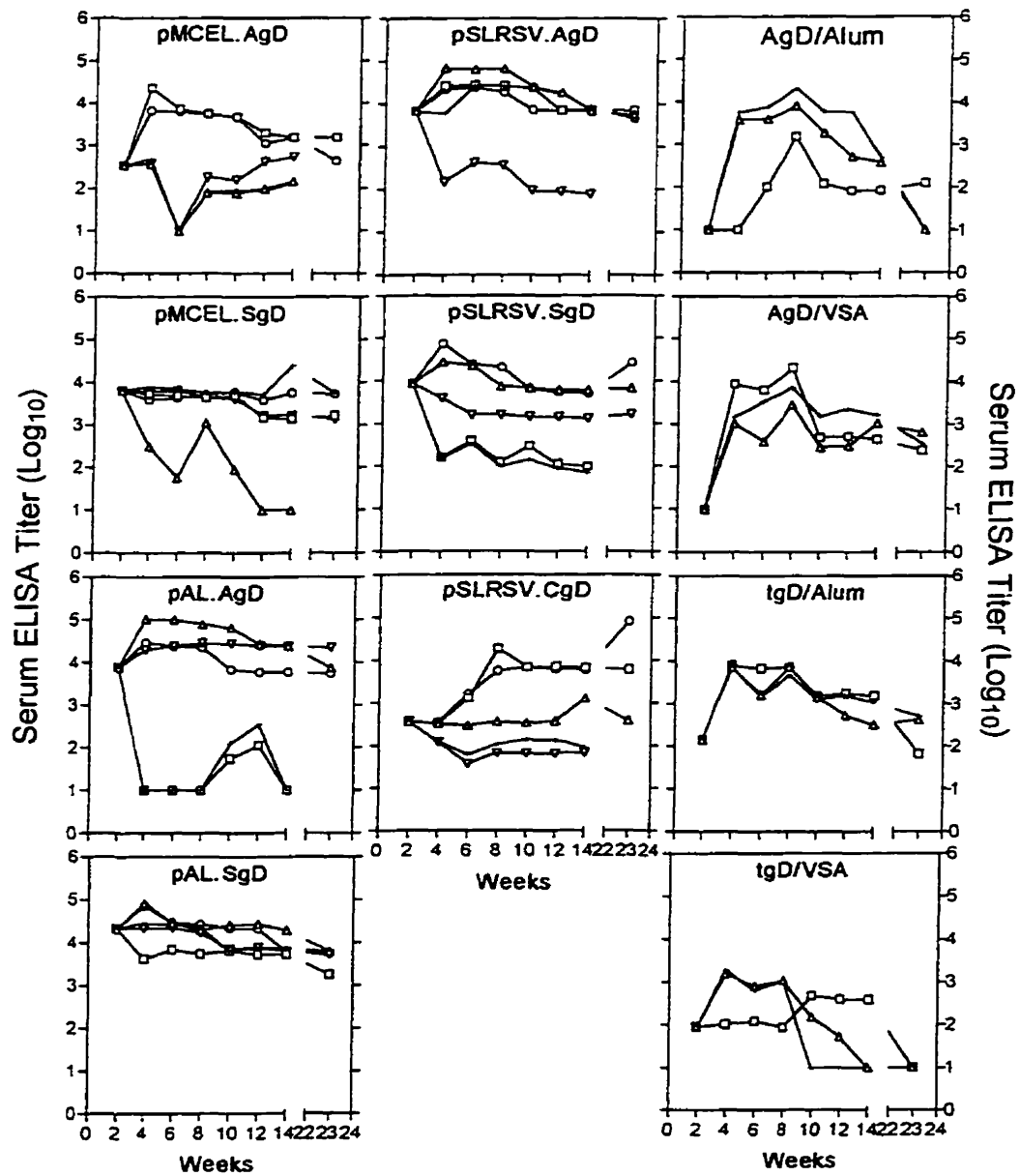


Table 3.2 Mean serum ELISA titers from seropositive mice at various times following immunization with DNA-based or conventional vaccines.

Vaccine Groups	# Seropositive Mice	Time (Weeks post immunization)		
		6	12	23
pAL.AgD	3	50,130 \pm 25,960	19,260 \pm 6,705	11,740 \pm 5,288
pAL.SgD	5	22,990 \pm 4,198	13,890 \pm 832	5,003 \pm 832
pMCEL.AgD	2	6,794 \pm 423	1,523 \pm 394	974 \pm 551
pMCEL.SgD	4	5,521 \pm 620	3,032 \pm 889	3,440 \pm 1,118
pSLRSV.AgD	4	36,340 \pm 9,824	12,540 \pm 3,642	5,415 \pm 535
pSLRSV.SgD	3	15,970 \pm 7,205	4,268 \pm 1,452	11,460 \pm 7,542
pSLRSV.CgD	2	1,077 \pm 401	4,567 \pm 2,105	29,350 \pm 26,210
AgD/Alum	3	3,829 \pm 2,146	2,157 \pm 1,862	48 \pm 38
tgD/Alum	3	3,244 \pm 1,698	1,284 \pm 375	331 \pm 134
AgD/VSA	3	3,372 \pm 1,708	1,039 \pm 632	403 \pm 116
tgD/VSA	2	752 \pm 333	152 \pm 121	10 \pm 0

Note: Values were obtained from mice depicted in Fig. 3.4 are represented with standard error (S.E.) of the mean.

3.4, and Table 3.2, clearly show that these conventional vaccine groups all displayed titers that were at background levels by 23 weeks. However, mean serum IgG titers in all seropositive DNA-vaccine groups were significantly ($p \leq 0.05$) above background at 23 weeks. Despite clear evidence of serum titers at 23 weeks post-immunization, mean titers from several DNA vaccine groups, including: pAL.AgD/SgD, pMCEL.AgD and pSLRSV.AgD, showed evidence of decline. Due to relatively low numbers of seropositive animals in some of these groups a statistical analysis on most groups was unwarranted. However, using a Mann-Whitney t-test it was shown that the decline in titers observed in 5 seropositive mice immunized with pAL.SgD at 23 weeks was significant ($p < 0.05$). Table 3.2 shows two DNA vaccine groups (pSLRSV.SgD and pSLRSV.CgD) with apparent increases in serum titers from 12 weeks to 23 weeks. These

values are the result of one mouse in the pSLRSV.SgD group and one in pSLRSV.CgD group displaying a 5 and 15 fold increase in serum IgG titer, respectively (data not shown). The remaining animals in each group maintained titer levels and showed no evidence of decline at 23 weeks.

Figs. 3.5 and 3.6 depict early serum IgG kinetics for two separate experiments in which mice were immunized using a split dose protocol (see methods). Fig. 3.5 shows, once again, the 2–4 week lag in serum titer development in mice immunized with plasmid encoding the cytosolic version of gD. It is also apparent that immunization with pSLRSV.CgD does not result in 100 % seroconversion at 4 or 6 weeks. Mice immunized with pSLRSV.AgD and pSLRSV.SgD tended to display 80 and 90% seroconversion efficacies at 2 weeks, respectively, with 100% seroconversion typically occurring by 4 weeks (Fig 3.5 and 3.6). One mouse immunized with pSLRSV.SgD did not show any gD specific titers at 4 weeks (Fig. 3.6), however, this mouse developed low, but detectable, titers by 6 week. There was no significant difference in mean titer values for mice immunized with either pSLRSV.AgD or pSLRSV.SgD, at 6 weeks. However, mice immunized with pSLRSV.CgD showed significantly lower mean titers than mice immunized with plasmids encoding the cell surface (pSLRSV.AgD) or secreted (pSLRSV.SgD) forms of gD (Fig. 3.5). Fig. 3.6 shows the early serum IgG kinetics for mice immunized subcutaneously with VSA/tgD. Only one mouse had seroconverted prior to boosting at 2 weeks. Surprisingly all 8 mice in this vaccine group had seroconverted by 4 weeks with a mean titer that was significantly lower ($p \leq 0.05$), than that observed in mice immunized with pSLRSV.AgD or pSLRSV.SgD. Typically, in our

Fig. 3.5 Early serum antibody levels after immunization with plasmids encoding different forms of BHV-1 gD. C3H/HeN mice were immunized with plasmids encoding cell associated (AgD or CgD) or secreted (SgD) antigens. These experiments represent serum ELISA titers determined as described for Fig. 3.4. Experimental groups had 10 mice per group and include a Null plasmid control group. Mice 6-7 weeks of age were immunized in each quadriceps muscle mass with 50 μ g DNA (total of 100 μ g). All mice were boosted at 2 weeks after initial immunization with the same dose. Plasmid construct designations are indicated in the top centre of each graph.

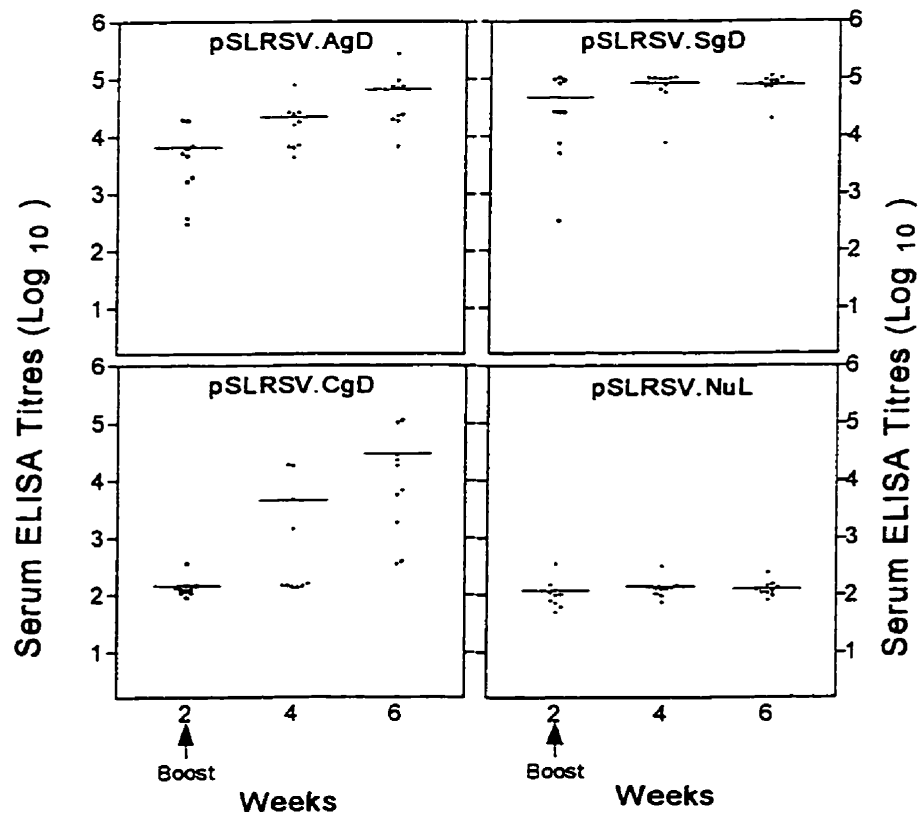
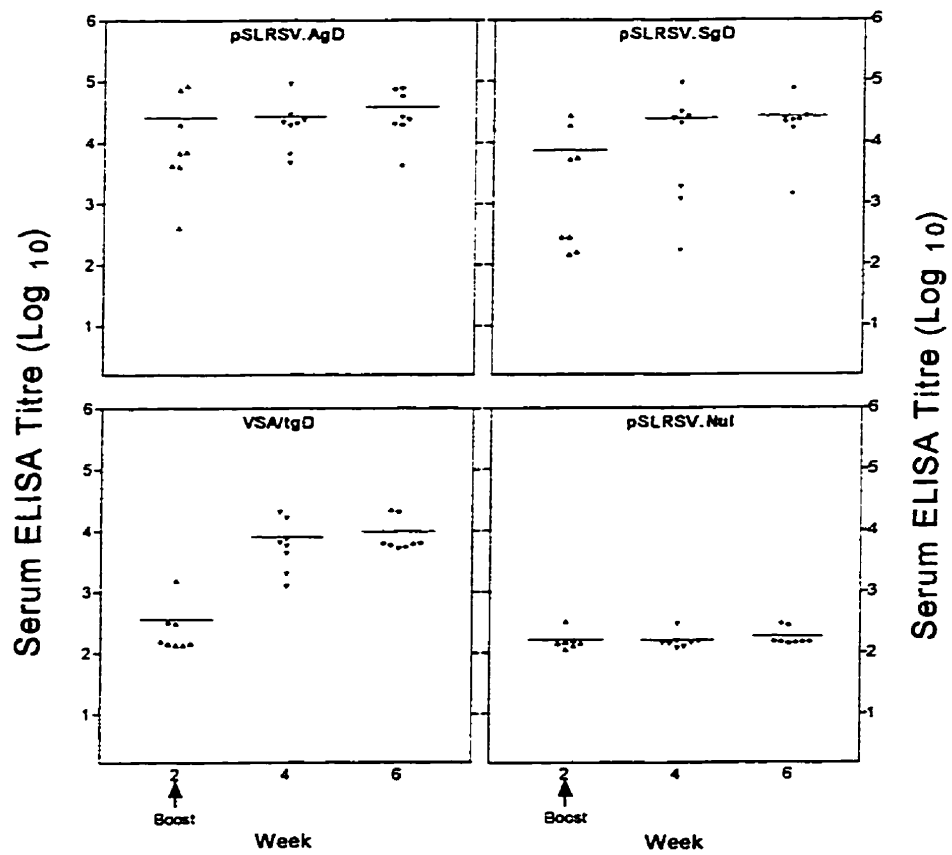


Fig. 3.6 Serum antibody levels in C3H/HeN mice immunized with plasmids encoding cell associated or secreted antigens. These experiments represent serum ELISA titers determined as described for Fig. 3.4. Experimental groups had 8 mice per group and include a conventional vaccine (VSA/tgD) control group. Mice 6-7 weeks of age were immunized in each quadriceps muscle mass with 50 μ g DNA (total of 100 μ g). Mice receiving conventional vaccines were immunized S.Q. with 400 η g affinity purified rtgD in 100 μ L VSA + HBSS. All mice were boosted at 2 weeks after initial immunization with the same dose. Plasmid constructs or conventional vaccines designations are indicated in the top centre of each graph.



hands, C3H/HeN mice are poor responders (50-60% seroconversion) to several different conventional adjuvants (Alum and VSA3) formulated with recombinant truncated BHV-1 gD. It is not known why all mice seroconverted in this one instance.

3.3.3 Serum IgG isotype in DNA-immunized mice.

To further characterize the serum antibody responses we determined the IgG isotype profiles in mice immunized with plasmids encoding each of three different forms of BHV-1 gD. Mice immunized with plasmids encoding the cell-associated versions of BHV-1 gD display a predominance of serum IgG_{2a}, while mice receiving plasmids encoding a secreted form of gD showed a predominance of IgG₁. Sera taken at 6 and 12 weeks post immunization, shown in Fig. 3.4, were assessed for serum IgG isotypes using an indirect ELISA. Calculation of IgG₁:IgG_{2a} ratios clearly demonstrate that mice immunized with plasmids encoding cell-associated versions of BHV-1 gD (pSLRSV.AgD and pSLRSV.CgD) show a predominance of IgG_{2a}, whereas mice immunized with plasmid encoding the secreted version of gD (pSLRSV.SgD) show a predominance of IgG₁ (Fig. 3.7). In two separate experiments this trend was observed to be reproducible and that the predominant isotype did not occur as the exclusive isotype (Figs. 3.8 and 3.9). Figs. 3.8 and 3.9 demonstrate that restriction of the plasmid encoded antigen to the transfected cell results in the predominance of IgG_{2a} in 100 % of seropositive mice immunized with pSLRSV.CgD and 86% of mice immunized with pSLRSV.AgD. Similarly, 95% of mice immunized with pSLRSV.SgD displayed a predominance of serum IgG₁ (Figs. 3.8 and 3.9). In addition it was observed that mice

Fig. 3.7 Serum IgG isotype ratios after immunization with DNA vaccines. Anti-BHV-1 gD IgG₁:IgG_{2a} isotype ratios in seropositive C3H/HeN mice 6 and 12 weeks after immunization with plasmids encoding cell associated (pSLRSV.AgD or pSLRSV.CgD) or secreted (pSLRSV.SgD) forms of BHV-1 gD. BHV-1 gD specific IgG₁ or IgG_{2a} antibody isotypes were detected by ELISA with biotinylated secondary antibodies. Sera was assayed from seropositive mice indicated in Fig. 3.4.

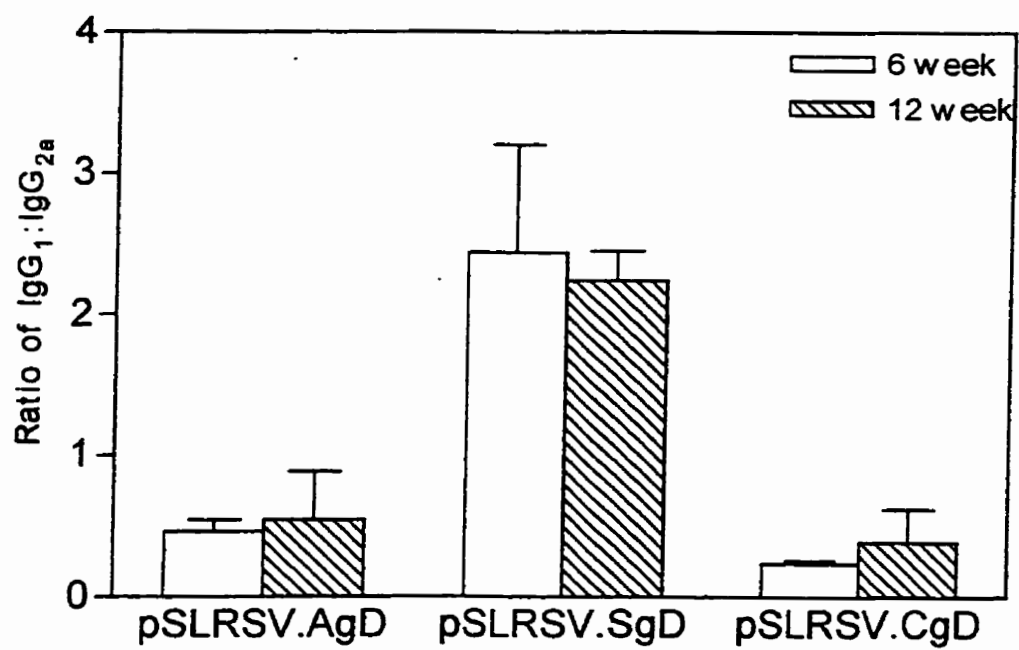


Fig. 3.8 Serum IgG isotype profiles in mice immunized with DNA vaccines. Data is from individual mice after intramuscular immunization with plasmids encoding cell associated (pSLRSV.AgD or pSLRSV.CgD) or secreted (pSLRSV.SgD) forms of gD. BHV-1 gD specific IgG₁ or IgG_{2a} isotypes were detected by ELISA using biotinylated secondary antibodies. Antibody titers were calculated as described in Fig. 3.4. Values are taken from mice described in Fig. 3.5 and depicted as $1/\log_{10}$ of antibody titer (determined by serial end-point dilution analysis).

Serum ELISA Titres

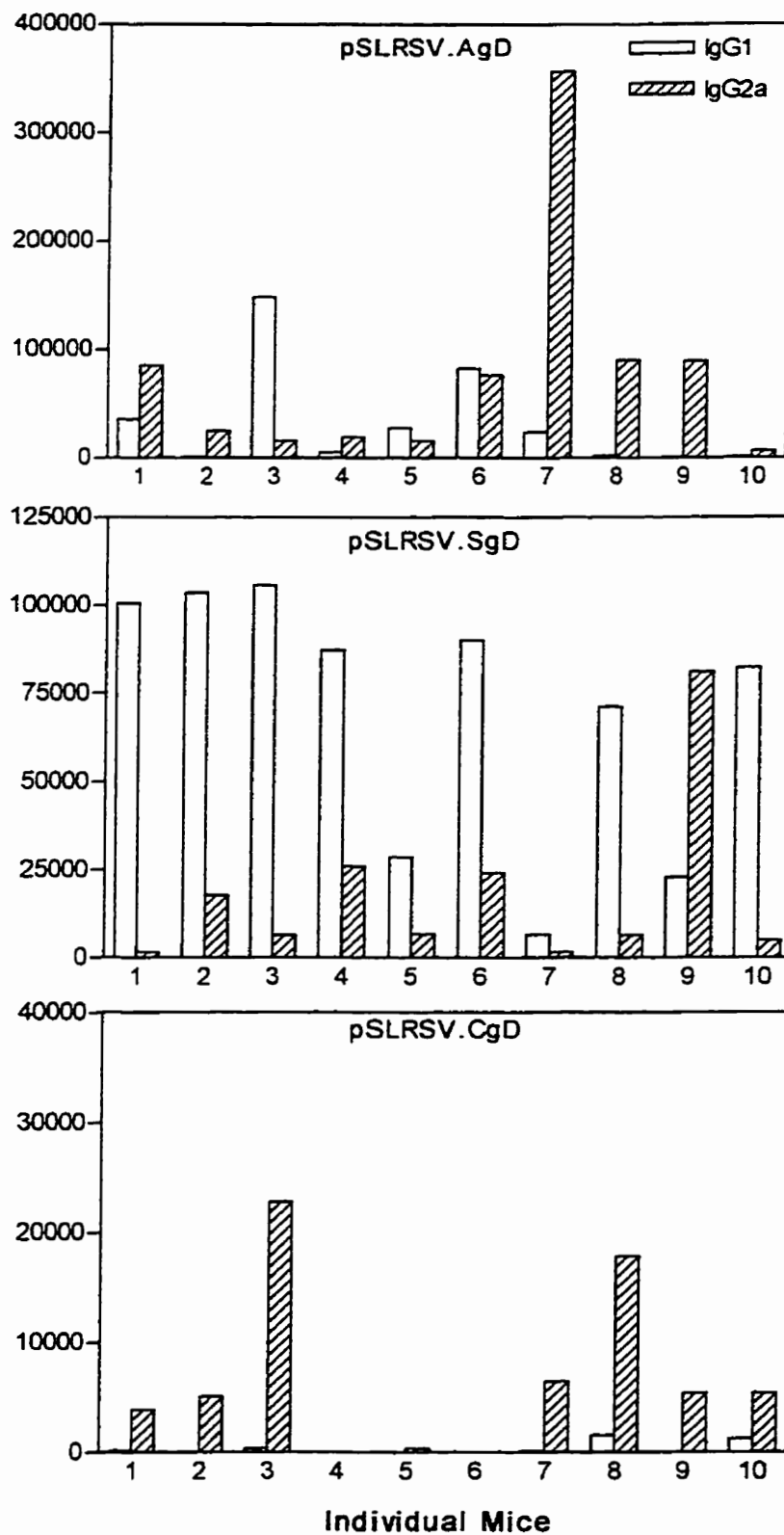
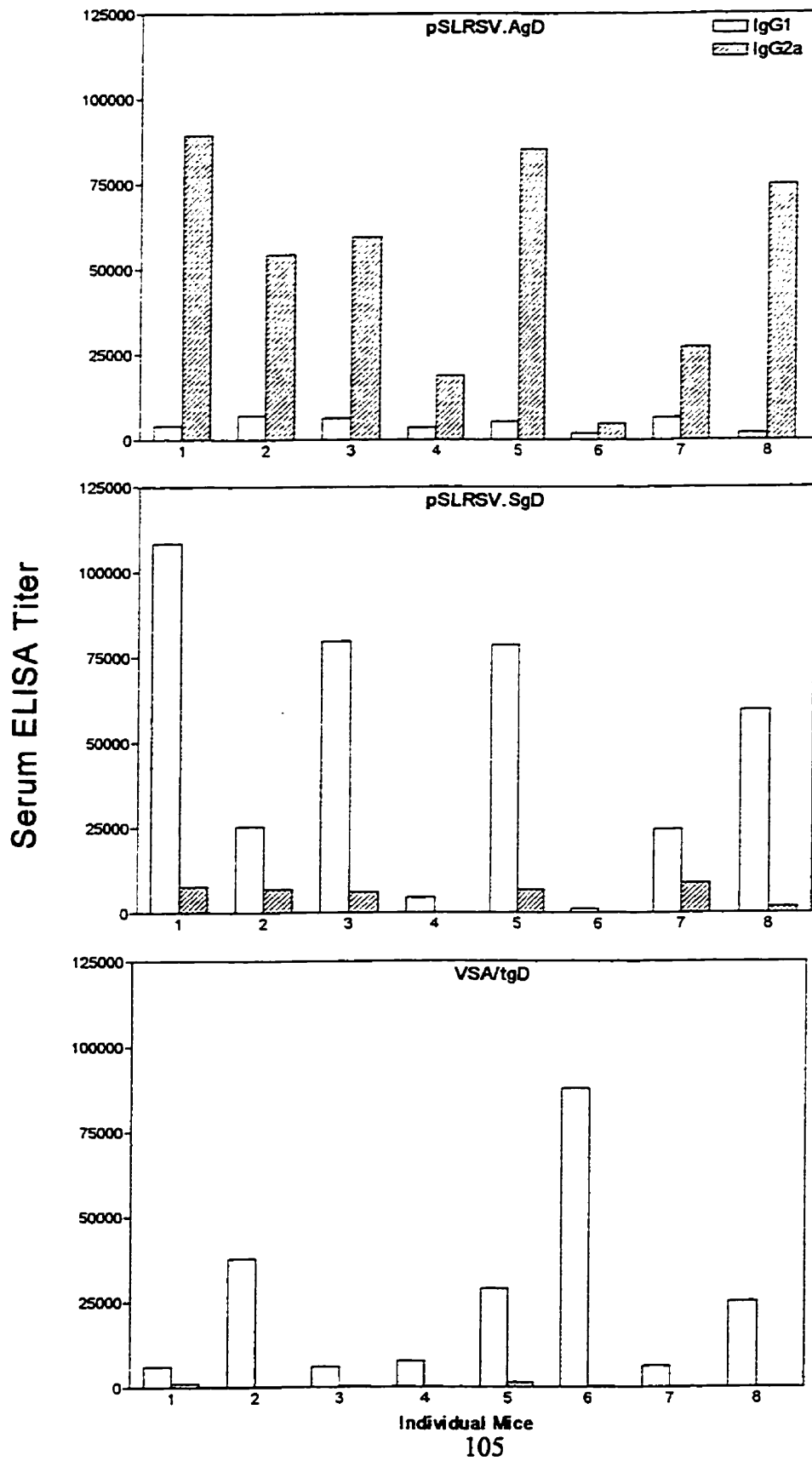


Fig. 3.9 Serum IgG isotype after immunization with pSLRSV.AgD or pSLRSV.SgD. Data represents individual mice 6 weeks after intramuscular immunization with DNA vaccines or subcutaneous immunization with recombinant truncated gD (tgD) formulated in VSA3. BHV-1 gD specific IgG₁ or IgG_{2a} isotypes were detected by ELISA using biotinylated secondary antibodies. Antibody titers were calculated as described in Fig. 3.4. Values are determined for mice described in Fig. 3.6 and depicted as $1/\log_{10}$ of antibody titer (determined by serial end-point dilution analysis).



immunized with pSLRSV.CgD showed higher levels of IgG_{2a}, relative to IgG₁, than mice immunized with pSLRSV.AgD.

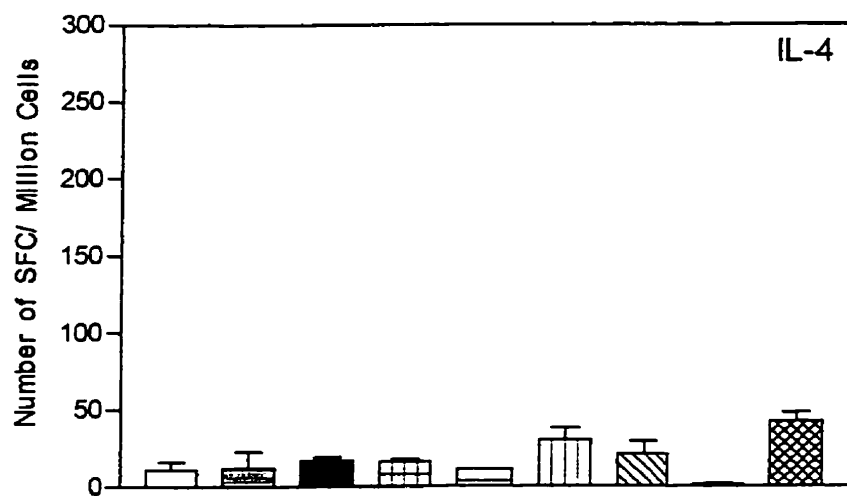
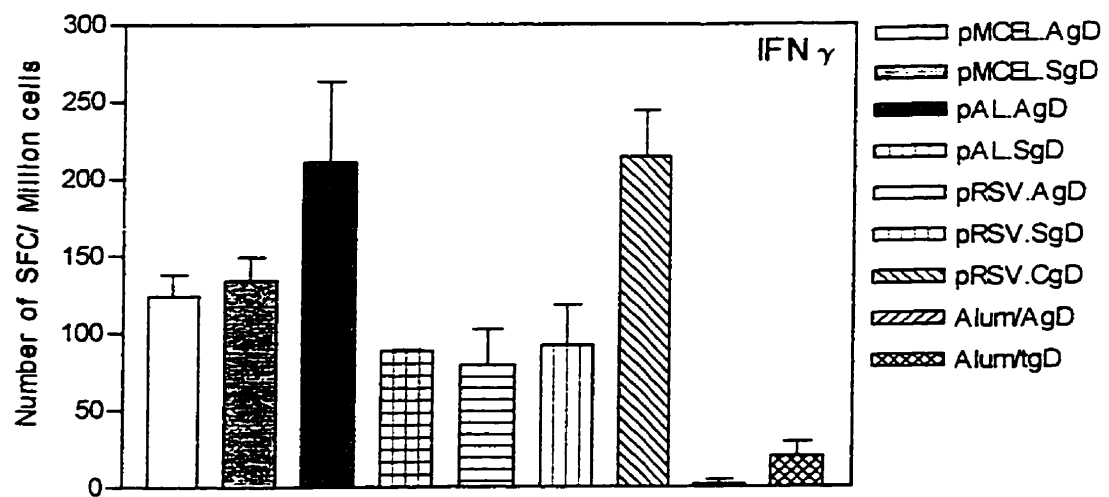
3.3.4 Splenic cytokine profiles reflect a Th1-like phenotype.

We assessed the cytokine profile in spleens harvested 6 months after the initial immunization of C3H/HeN mice with DNA-based or a conventional vaccine and found that splenic cytokine ELISPOT profiles did not correlate to the predominant serum IgG isotype. Following *in vitro* restimulation for 40 hours, we determined the number of IL-4 and IFN γ secreting splenocytes (Fig. 3.10). In all groups of mice immunized with plasmid DNA encoding authentic or deletion mutants of BHV-1 gD the exclusive predominance of IFN γ secreting cells was observed. Mice immunized with a conventional Alum formulation that had incorporated either purified viral authentic gD or recombinant truncated gD showed either no cytokine production (Alum/AgD) or a predominance of IL-4 (Alum/tgD).

3.3.5 The Iliac lymph node drains the deep muscles of the murine thigh.

Prior to characterizing cytokine and B-cell IgG isotype profiles within draining lymph nodes we felt it was paramount to ensure that the predominant draining node was identified. At least one recent paper described lymph node harvests and cytokine profiles from a pool of lymph nodes that included the mesenteric and superficial inguinal nodes (Mor et al., 1995). We were unconvinced that these nodes were the predominant draining nodes and demonstrated, using several approaches, that the iliac lymph node was the

Fig. 3.10 Splenic cytokine profiles in mice 5 1/2 months post-immunization. Seropositive mice depicted in Fig.3.4. were assessed for antigen specific production of IFN γ or IL-4. Spleens from seropositive mice were pooled and stimulated, *in vitro*, for ~ 40 hours with affinity purified BHV-1 gD. *In vitro* stimulated splenocytes were plated at 1.0×10^6 or 0.5×10^6 cells/well on Polyfiltronics plates. Cytokine secreting splenocytes are depicted as the number of spot forming cells (SFC) per million plated cells. Mean values of triplicate wells are depicted.



primary draining node of the deep muscles of the thigh. Euthanasia of C3H/HeN mice 5 minutes following an i.m. (quadriceps) injection of a 1% Evans Blue solution clearly showed dye uptake only in the iliac node (Fig. 3.11). Careful dissection showed that a substantial amount of dye also occurred within the subcutaneous fatty tissue occurring in the inguinal region of these dye injected mice and that, despite the presence of this dye, the inguinal node, which is found embedded in this fatty tissue, remained relatively unstained (Figs. 3.11, A and B). We felt that reflux of injected material back along the needle tract was the most likely explanation for the presence of blue dye in the subcutaneous inguinal region.

We also utilized PCR to detect the presence, or absence, of BHV-1 gD encoding sequences using primers described in materials and methods. Surprisingly, a 435 bp gene fragment was identified by PCR in not only the iliac lymph node, but also the inguinal node (Fig. 3.12). In both instances gD specific sequences were identified at 20 minutes post injection but not at 20 hours. BHV-1 gD specific sequences in the superficial inguinal node may have occurred in one of several manners, including: contamination of the node tissue sample with adjacent contaminated fatty tissue, or access to the node as a result of drainage of the adjacent contaminated fatty tissue. Despite the equivocal PCR data, we felt that the iliac node was indeed the primary draining node of the deep muscles of the thigh and that post injection healing of the needle tract followed by antigen production by transfected myocytes would drain to the iliac node and not the superficial inguinal node. Furthermore, immunological data from both iliac and inguinal nodes, following immunization with pSLRSV.AgD and pSLRSV.SgD, supports this conclusion

Fig. 3.11 Identification of iliac lymph node as primary draining node for the deep muscles of the murine thigh. Mice were sacrificed 5 minutes after an intramuscular injection of 1% Evans blue dye into the quadriceps muscle mass. The iliac lymph node is identified by an arrow (**Fig. 3.11, A**). **Fig. 3.11, B** shows blue stained iliac nodes flanking an unstained superficial inguinal node after removal from the same mouse.

A

1.0 cm



B

1.0 cm

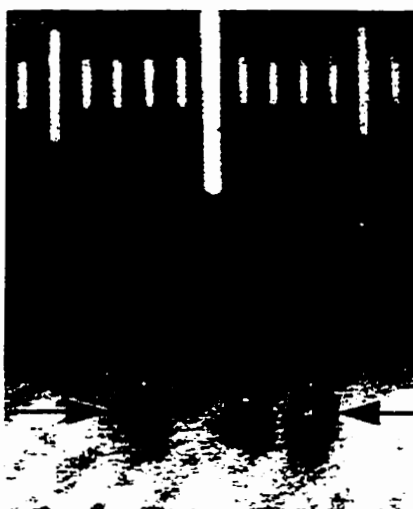
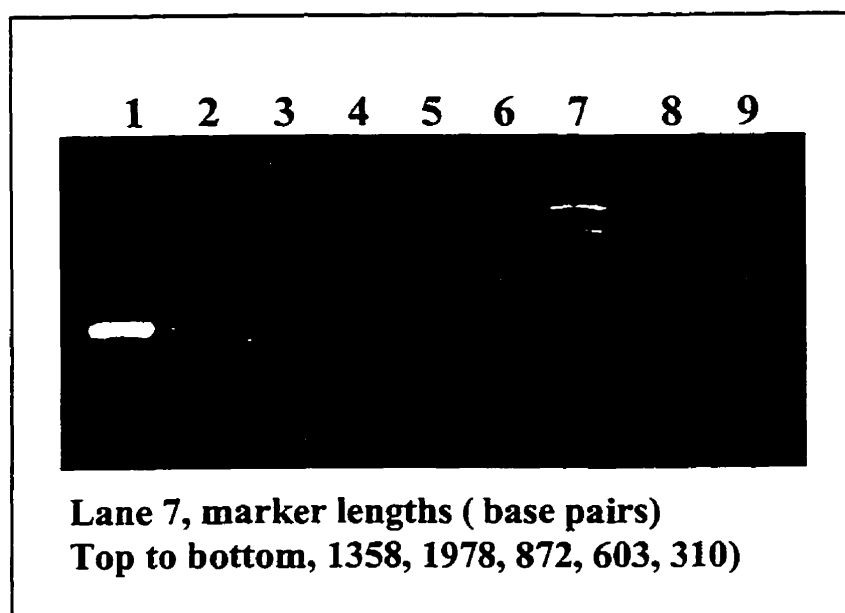


Fig. 3.12 Injected plasmid DNA is detectable by PCR in two lymph nodes. DNA purified from the iliac and superficial inguinal lymph nodes show that injected DNA gains access to these sites shortly after injection. Mice were injected with 100 µg of pSLRSV.AgD and sacrificed 20 minutes or 20 hours post-injection. Purified DNA was screened by PCR for the presence of BHV-1 gD sequences. Lane 1, plasmid pSLRSV.AgD positive template control; 2, inguinal node 20 minutes post-injection; 3, inguinal node 20 hours post-injection; 4, iliac node 20 minutes post-injection; 5, iliac node 20 hours post-injection; 6, iliac node 20 minutes following injection with pSLRSV.Nul vector; 7, φX-174 DNA-Hae III marker digest (Pharmacia) (base pair number of first five bands from top to bottom: 1358, 1078, 872, 603, 310); 8, pSLRSV.Nul template negative control; 9, pSLRSV.AgD positive template control.



(Fig. 3.13).

3.3.6 Cytokine and antibody forming cell profiles in the iliac node.

The draining lymph node cytokine and B-cell isotype ELISPOT profiles correlate to the predominant serum isotype. Fig. 3.13 demonstrates that early splenic cytokine kinetics still show a predominance of IFN γ secreting cells. There appears to be a predominance of IFN γ secretion occurring in mice immunized with a conventional vaccine (VSA3:tgD), however, this value becomes negligible when background cytokine production is subtracted (see Null plasmid group in Fig. 3.13). Subtraction of Null group IFN γ levels from the displayed inguinal lymph node cytokine secreting cell numbers suggests that little antigen-specific immunological responses are occurring in this nondraining node at 7 weeks post immunization. The iliac lymph node shows a predominance of IFN γ secreting cells in mice immunized with the cell-associated pSLRSV.AgD, and a significant increase in the level of IL-4 secretion in mice immunized with pSLRSV.SgD. Upon subtraction of the IFN γ counts from the Null plasmid group the level of IL-4 production becomes significantly greater than the number of antigen-specific IFN γ secreting cells in the pSLRSV.SgD vaccinated group. There was a significant level of IL-4 production in restimulated inguinal node cells taken from mice immunized subcutaneously with VSA3/tgD. B-cell isotype ELISPOT data from mice immunized with a DNA-based, or a conventional (VSA3/tgD) vaccine, is illustrated in Fig. 3.14. Numbers of splenic antibody forming cells (AFCs) are either absent or below detection limits in all groups except mice immunized with pSLRSV.AgD. The

Fig. 3.13 Cytokine profiles in lymph nodes and the spleen after immunization with DNA vaccines. Antigen specific cytokine profiles were measured in several different lymphoid tissues of C3H/HeN mice immunized with plasmids encoding cell associated or secreted version of BHV-1 gD. Seropositive mice were euthanized 5 weeks after boosting and spleens, draining and nondraining lymph nodes were excised and pooled. Pooled cell populations were stimulated *in vitro* for 18-20 hours with 400 ng affinity purified BHV-1 gD. Following *in vitro* stimulation cells were harvested and plated on Polyfiltronics plates at 1.0×10^6 cells/well in triplicate. Pooled values are represented as mean values and as number of cytokine secreting cells per million plated cells. Plate control wells for nonspecific binding of cytokine were set up for all groups and mean values were subtracted from all groups.

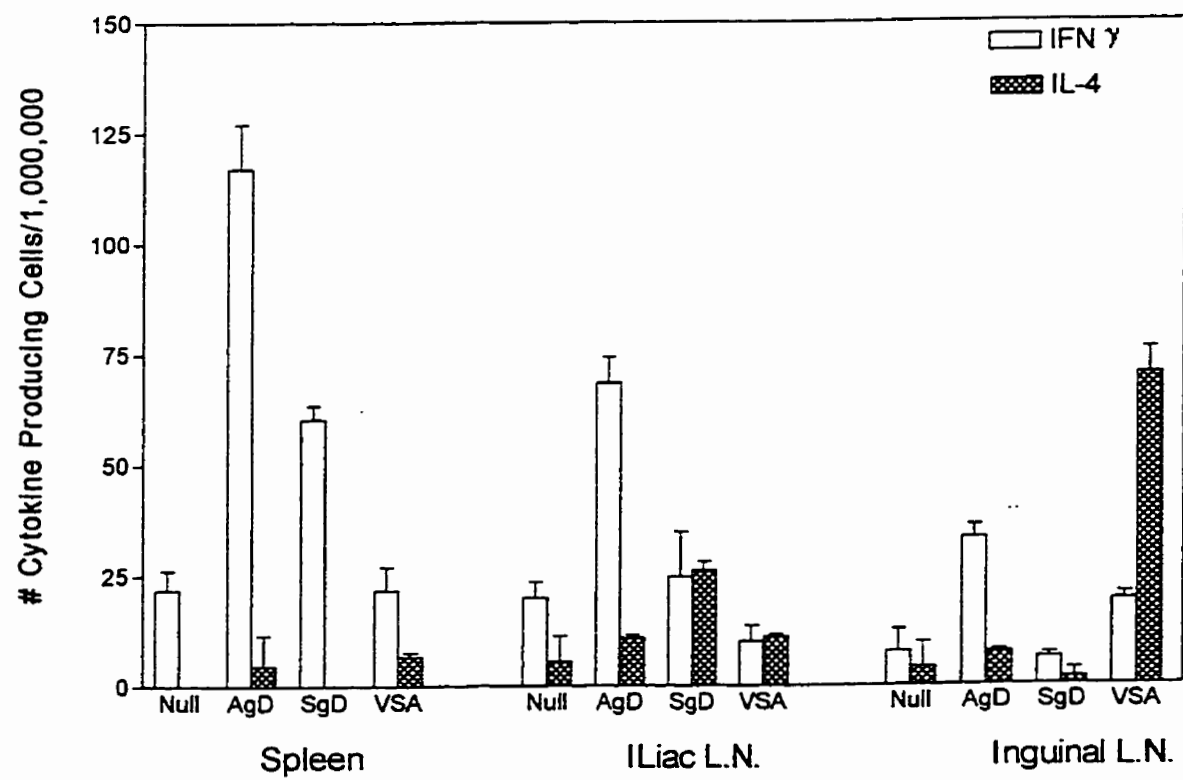
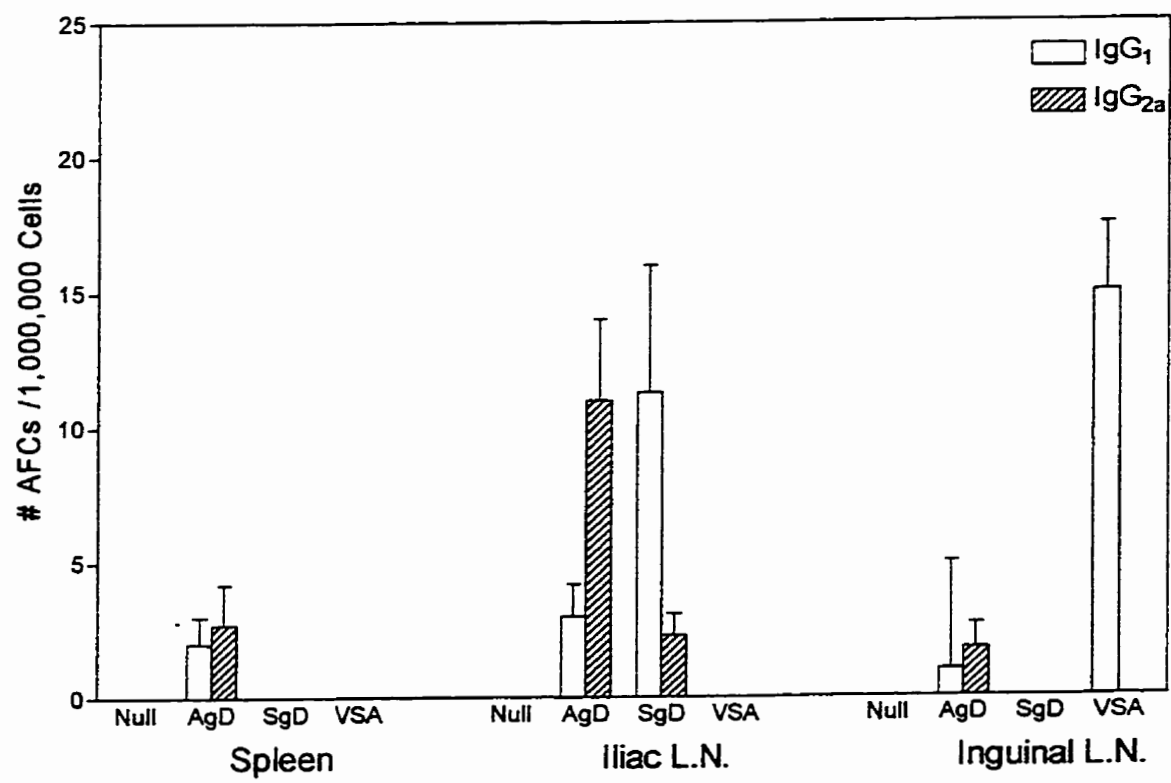


Fig. 3.14 Number of antibody forming cells secreting IgG₁ or IgG_{2a} after immunization with DNA vaccines. Data depicts antigen specific IgG₁ and IgG_{2a} production in several different lymphoid tissues of C3H/HeN mice immunized with plasmids encoding cell associated or secreted version of BHV-1- gD. Seropositive mice were euthanized 5 weeks after boosting and spleens, draining and nondraining lymph nodes were excised and pooled. Following a collagenase/DNaseI dissociation digestion pooled cell populations were plated at 1×10^6 cells/well on Millipore ELISPOT plates, in triplicate. Pooled values are represented as mean number of Ig secreting cells per million plated cells. Plate control wells for nonspecific binding of immunoglobulin isotypes were set up for all groups and mean values were subtracted from all groups.



total number of AFCs, in the spleens of mice immunized with pSLRSV.AgD would amount to approximately 200 - 250/spleen. The IgG isotype of AFCs taken from the iliac lymph node correlated well with the predominant cytokine ($\text{IFN } \gamma \rightarrow \text{IgG}_{2a}$, $\text{IL-4} \rightarrow \text{IgG}_1$) secreted within this node and with the predominant serum IgG isotype. Mice immunized with plasmid DNA encoding the cell-associated gD (pSLRSV.AgD) displayed a predominance of IgG_{2a} while mice immunized with plasmid encoding the secreted form of gD (pSLRSV.SgD) displayed a predominance of IgG_1 . The subcutaneously occurring inguinal lymph node showed a substantial and exclusive number of IgG_1 secreting AFCs taken from mice immunized with VSA3:tgD.

3.3.7 DNA vaccines elicit viral neutralizing antibodies.

Serum antibodies taken from mice immunized with plasmids encoding the authentic or secreted versions of BHV-1 gD were able to neutralize virulent BHV-1 *in vitro*. Individual immune sera from mice 7 weeks after an initial immunization (5 weeks post boost) with 100 μg plasmids: pSLRSV.Nul, pSLRSV.AgD, pSLRSV.SgD and pSLRSV.CgD showed that only mice immunized with plasmids encoding the authentic and secreted versions of gD were able to effectively neutralize virulent BHV-1 (Fig. 3.15). In this assay there was no significant advantage in neutralizing capacity noted between groups immunized with pSLRSV.AgD or pSLRSV.SgD.

3.3.8 Mouse strain affects serum isotype profile.

We wished to determine if mouse strain had an impact on the serum IgG isotype

profile following immunization with different forms of plasmid encoded BHV-1 gD. We found that IgG₁ was the predominant serum isotype in C57BL/6 (H-2^b) mice immunized with either pSLRSV.SgD or pSLRSV.AgD. Fig. 3.16 illustrates mean IgG isotype titers occurring in C57BL/6 mice immunized with the pSLRSV.AgD (cell-associated antigen) or pSLRSV.SgD (extracellular antigen). Surprisingly the serum isotype trend observed in C3H/HeN (H-2^k) did not manifest itself in C57BL/6 mice. Antigen-specific, total IgG serum antibody kinetics mirrored those seen in C3H/HeN, however, the predominant serum isotype was, in most animals, IgG₁. Despite the apparent predominance of serum IgG₁, as indicated by mean isotype titers in these mice, there was clear evidence that mice receiving pSLRSV.AgD (cell-associated gD) did develop significantly higher IgG_{2a} titers than mice immunized with pSLRSV.SgD. Indeed three mice (of eight) in the pSLRSV.AgD group showed IgG_{2a} serum titers equivalent to, or greater than, the IgG₁ titers. Also, the IgG₁:IgG_{2a} ratio dropped from 85:1 (pSLRSV.SgD) to 4.4:1 (pSLRSV.AgD) and this difference was due, in large part, to the significant increase in IgG_{2a} levels in mice immunized with pSLRSV.AgD (data not shown). Thus, although C57BL/6 mice did not show the serum isotype polarity existing in C3H/HeN mice immunized with plasmids encoding either the cell-associated or extracellular versions of gD, the tendency for the cell-associated version of gD (pSLRSV.AgD) to skew serum isotype responses towards greater levels of IgG_{2a} was reproduced.

3.4 Discussion

DNA-based vaccines offer a number of potential advantages over conventional

Fig. 3.15 Functional differences in serum antibodies after immunization with plasmids encoding different forms of BHV-1 gD. Serum antibody responses following immunization and boost (2 weeks) of mice with plasmids encoding cell membrane anchored gD (pSLRSV.AgD), secreted gD (pSLRSV.SgD) cytoplasmic gD (pSLRSV.CgD), or Null vector (pSLRSV.Nul). gD-specific ELISA titers are expressed as the reciprocal of the extrapolated (see Methods) dilution resulting in three standard deviations above the control value (prebleed sera). BHV-1 neutralizing antibody titers are expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaques relative to virus control. Results are expressed as geometric mean and bars indicate the standard error of 4 (pSLRSV.CgD and pSLRSV.Nul), 8 (pSLRSV.SgD), or 9 (pSLRSV.AgD), mice.

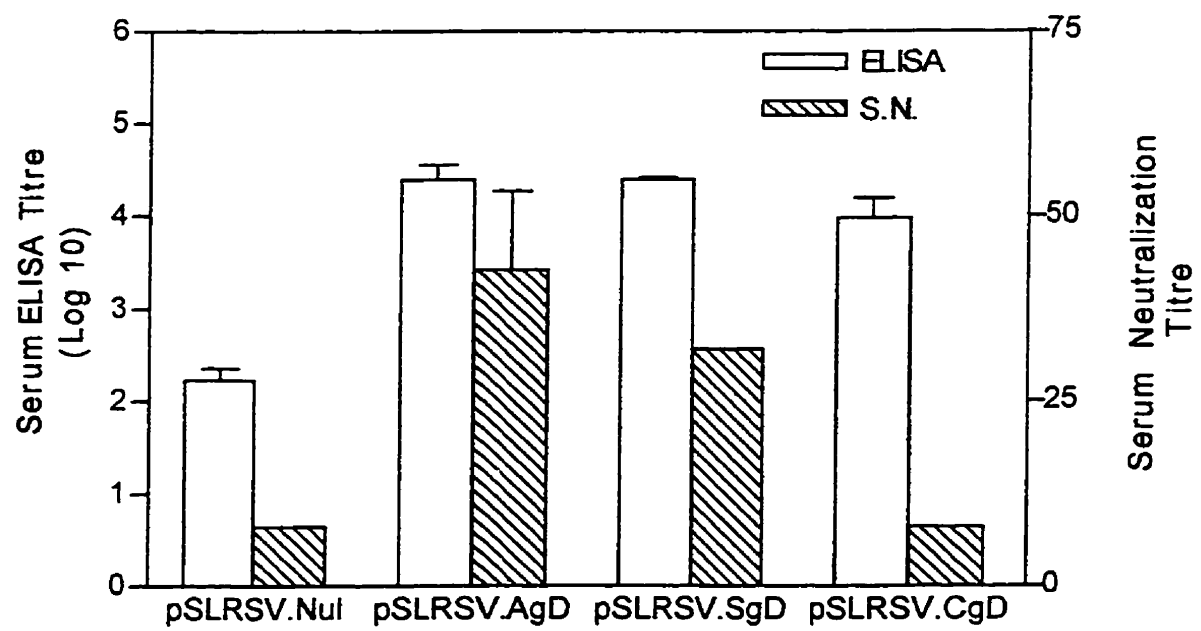
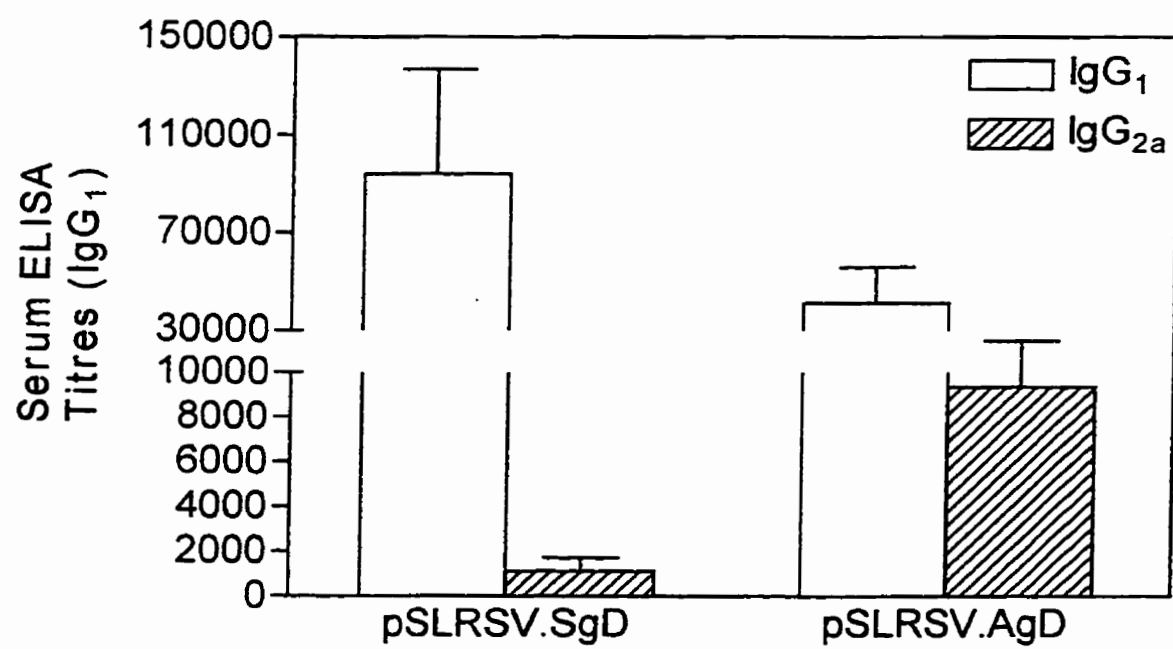


Fig. 3.16 Serum antibody profiles in C57BL/6 mice after immunization with DNA vaccines. Serum antibody isotype ELISA titers for C57BL/6 mice immunized intramuscularly with plasmids encoding the anchored (pSLRSV.AgD) or secreted (pSLRSV.SgD) version of BHV-1 gD. Isotype titers represented above were determined from bleeds harvested 6 weeks post immunization. The differences between IgG₁ titers was not statistically different ($p < 0.05$), however, the difference between IgG_{2a} titers was found to be statistically different ($p < 0.05$).



vaccines that include: breadth of efficacy (single dosing, long lasting immunity, both cell-mediated and humoral immunity, and the potential to polarize immune responses) reduced downstream processing, and vaccine stability (Donnelly et al., 1993; Babiuk et al., 1996; Ertl and Xiang, 1996; Ulmer et al., 1996a; Ulmer et al., 1996b). Among these advantages it would seem that duration and the protective efficacy of the resulting immune responses are of the utmost significance when consideration is given to the development of vaccines. We demonstrated, using a moderate strength RSV-LTR regulatory element, that serum IgG ELISA titers in excess of 100,000 were achievable and that significant levels of antigen-specific antibody were still evident 23 weeks post immunization. We also demonstrated that the cellular compartment in which the plasmid encoded antigen was expressed could profoundly influence the ensuing immune character.

Evidence in most mice, immunized with plasmids encoding secreted and membrane anchored versions of BHV-1 gD, suggested a decline in anti-gD serum IgG titers with time. However, there was also clear evidence of serum titer maintenance, and in some instances increases, in several mice immunized with plasmids encoding the intracellular or secreted version of gD at 23 weeks post immunization. These observations lend credibility to the suggestion that DNA-based vaccines have the potential to elicit long term humoral immune response. However, in mice immunized with the intracellular version of gD, the trade off for a durable humoral immune response appears to be a lag in serum antibody appearance and lower overall serum antibody titers. It also seems apparent that neutralizing serum antibody titers raised against this cytosolic,

nonglycosylated antigen, were lower than those occurring in mice receiving the appropriate post-translational modifications of BHV-1 gD (Tikoo et al., 1993). Indeed, it has been demonstrated previously that recombinant gD produced in *E. coli* generate excellent antibody titers when used to immunize cattle, however, serum neutralizing titers are nonexistent (van Drunen Littel-van den Hurk et al., 1993b). It was also hypothesized that the inability to induce good serum neutralizing titers was likely due to the lack of N-linked glycans at one of two glycosylation sites on the full length glycoprotein (Tikoo et al., 1993). The immunological significance of systemic antibody responses to antigens not normally occurring at the surface of pathogens or host cells (nonstructural proteins) is not well understood. However, there is recent evidence that humoral responses against nonstructural viral proteins of Yellow Fever, Dengue, murine hepatitis and rabies viruses, do confer significant levels of protection to the respective viruses (Nakanaga et al., 1986; Schlesinger et al., 1986; Lodmell et al., 1993; Slifka and Ahmed, 1996). Thus, the potential for more durable humoral responses and protective immunity may outweigh the apparent negative impact of intracellular antigens, delivered as DNA-based vaccines, on the magnitude and onset of serum IgG titers.

Several studies have been carried out that have addressed the longevity of humoral immune responses following immunization with DNA-based vaccines (Rhodes et al., 1993; Raz et al., 1994; Michel et al., 1995). A single i.m. injection of human cytomegalovirus (HCMV) immediate-early promoter driven genes encoding the major middle (preS2 and S) and large (preS1, preS2 and S) envelope proteins, as well as, plasmids encoding the major and middle envelope proteins of Hepatitis B virus achieved

serum ELISA titers between 10,000 and 50,000 in C57BL/6 (H-2^b) mice (Michel et al., 1995). Mice immunized with plasmids encoding the major middle and large envelope proteins of Hepatitis B virus showed some evidence of decline in serum antibody levels at 24 weeks, however, animals immunized with plasmids encoding only the major envelope protein showed no evidence of decline of serum IgG levels over the same time period. Balb/c (H-2k^d) mice injected i.m. with a single dose of plasmid utilizing regulatory elements from the RSV-LTR and encoding the influenza nucleoprotein (NP) antigen resulted in excellent serum antibody titers that persisted, in at least 75% of mice, with little decay until 5 1/2 months post immunization (Rhodes et al., 1993). However, a consistent subpopulation of these mice developed peak titers between 2 and 3 months followed by a relatively rapid decay in titers. An explanation for this observed divergence in serum titer kinetics is not immediately forthcoming. However, we cannot rule out the possibility that CD8⁺ cytotoxic T-cells directed against host cells expressing foreign antigen are responsible for clearing an important source of antigen (Davis et al., 1997). Finally, Balb/c mice injected i.d. with plasmids encoding the influenza NP gene under the control of the HCMV immediate-early promoter in the context of the HCMV intron A regulatory element showed maintenance of serum titers for 68 weeks (Raz et al., 1994).

We do not yet know if lifelong immunity will be a ubiquitous feature of DNA-based vaccines that are expected and able to elicit efficacious humoral immune responses. It was hypothesized that long term humoral responses were a likely result of demonstrated *in-vivo* persistence of plasmid DNA and reporter gene expression in mice

(Wolff et al., 1992). It now seems apparent that long term, extrachromosomal, persistence and expression of encoded genes may be largely dependent on the encoded antigen. For example, a recent study utilizing the reporter gene luciferase clearly demonstrated a decline to background 8 weeks following injection (Wolff et al., 1990; Norman et al., 1997). While this recent work suggests plasmid persistence and encoded gene expression can be highly variable it does not provide any insight into the mechanistic (extrachromosomal plasmid instability, cell toxicity, or antigen-specific cytotoxicity), causes responsible for the reduction in expression. Surprisingly our preliminary research suggested that a more durable humoral immune response occurred if the antigen was "restricted" to the intracellular compartment (Fig. 3.4, pSLRSV.CgD). Obviously, for a B-cell response to occur antigen must appear extracellularly. However, as with other studies demonstrating humoral immunity to intracellular antigens, the specific mechanism whereby cytosolic antigens gain access to the extracellular space is unknown (Ulmer et al., 1993; Kuhober et al., 1996; Geissler et al., 1997). One could hypothesize that nonspecific and/or specific cytolytic activity by NK cells and CD8⁺ CTLs may be responsible for an intermittent release of a "bolus" of cytosolic antigen and that these pulses of antigen are effective at "charging" follicular dendritic cells with immune complexes thereby maintaining long term serum titers (Gray and Skarvall, 1988; Bachman et al., 1994; Karre, 1995; Bachman et al., 1996; Helander et al., 1996). Certainly, recent evidence describes inflammatory reactions at the sites of DNA injection and supports the contention that *in vivo* transfected cells can become immunological targets as antigen-specific immunity develops (Davis et al., 1997). We have not been able

to identify specific major histocompatibility (MHC)-I restricted epitopes within BHV-1 gD in several mouse strains. While this phenomenon does not completely rule out the existence of antigen-specific CD8⁺ CTL activity, it suggests that in this particular instance durability of the humoral response may be a feature of diminished CTL activity unique to BHV-1 gD in mice.

Interestingly, the cell compartment in which the antigen was expressed had little impact on the character of the splenic T-cell cytokine profile. Initial experiments demonstrated an almost exclusive production of IFN- γ from *in vitro* stimulated splenocytes taken from C3H/HeN mice 21 weeks after boosting (Fig. 3.10). This data is consistent with current published research demonstrating a Th1-type of immune response to many i.m. or i.d. administered DNA-based vaccines (Donnelly et al., 1993; Ertl and Xiang, 1996; Hassett and Lindsay Whitton, 1996; Ulmer et al., 1996a; Ulmer et al., 1996b). The tendency for DNA-based vaccines administered i.m. or i.d to skew the immune response towards a Th1-type of responses is significant because protective immune response to many viral, Gram-negative bacterial, and some parasitic diseases are characterized by a potent Th1 type of immunity (Coutelier et al., 1988; Peterson et al., 1992; Scott, 1993; Nguyen et al., 1994; Mahon et al., 1995; Brubaker et al., 1996; Mosmann and Sad, 1996; Pope et al., 1996; Waris et al., 1996; Fresno et al., 1997; Gause et al., 1997). Splenic cytokine profiles may reflect the potency of cell-mediated immunity, when measured, CD8⁺ cytotoxicity. However, splenic cytokine profiles do not appear to accurately reflect the serum antibody character (Figs. 3.7, 3.8, 3.9, 3.10, 3.13 and 3.14).

It is paramount that problems associated with reliance on splenic cytokine profiles

and adherence to dogma describing cytokine involvement in isotype switching be considered when assessing immune responses in mice (Snapper and Paul, 1987; Snapper and Mond, 1993). The serum isotype profiles represent the sum of Ig secretion from plasma cells upon exit from the spleen and the draining lymph nodes (Slifka and Ahmed, 1996). Despite the prevalence of IFN- γ production in spleens of immune mice, and the overwhelming tendency to declare this a Th1-type immune response, we found that the expected preponderance of IgG_{2a} occurred only in mice immunized with plasmids encoding cell-associated gD (cytosolic or membrane anchored) (Figs. 3.7, 3.8, 3.9 and 3.10). Surprisingly, despite the predominance of IFN- γ produced in restimulated splenocytes, mice immunized with plasmids encoding the secreted form of gD consistently showed serum isotypes that were predominantly IgG₁ (Figs. 3.7, 3.8, 3.9 and 3.10). This immunoglobulin isotype dominated the serum IgG profile consistently over several different time points during three separate experiments (data not shown). It is becoming increasingly evident that despite clear evidence that IFN- γ and IL-4 play important roles in the generation of IgG_{2a} or IgG₁, respectively, the mechanistic routes to a specific Ig isotype are more complex than the singular presence, or absence, of either of these two cytokines (Coutelier et al., 1988; Kuhn et al., 1991; Nguyen et al., 1994; Coffman and von der Weid, 1997). However, in order to fully investigate the T and B-cell phenotype in mice receiving plasmids encoding secreted or cell-associated forms of BHV-1 gD we used ELISPOT to quantify the antigen-specific Ig isotype and cytokine profile within the primary draining lymph node. We found an antigen-specific elevation of IL-4 and IgG₁ secreting B-cells in the iliac lymph nodes of mice receiving this

construct as compared to mice receiving the plasma membrane anchored gD (Fig. 3.13). Thus, the draining lymph node cytokine and IgG isotype character may prove to more accurately correlate to the systemic immune character, at least at the time points assessed, than splenic immune responses. This apparent dichotomy between splenic cytokine profiles and those seen in draining lymph nodes, and in the context of published data describing kinetic differences in developing cellular immune repertoires in spleens, lymph nodes and bone marrow, underscore the importance of recognizing the temporal compartmentalization of immune responses to single antigens (Justewicz et al., 1995; Mo et al., 1995; Boyle et al., 1996) . Compartmentalization of immune responses is not a novel concept. However, the mechanism whereby a given antibody isotype predominates within a single, secondary lymphoid tissue remains unresolved. Historically, evidence suggested that the predominant, post pathogen/antigen exposure, cytokine profile within a given lymphoid tissue acts in a cognate and noncognate fashion to determine the B-cell immunoglobulin isotype (Coutelier et al., 1988). More recently, this hypothesis has been challenged with data showing simultaneous development of divergent, antigen-specific, antibody isotypes within the same draining lymph node (Sangster et al., 1997). In this instance it was hypothesized that noncognate activity by lymphoid cytokines could not be occurring and that dendritic cell cofactors, reflecting the anatomical source (mucosal versus subdermal) of pathogen, determined the isotypic outcome (IgA versus IgG2a). This information is not without precedence, for it has been demonstrated that dendritic cells from different tissues are able to facilitate Th2 or Th1-type immune responses that are dependent on the tissue of origin (Everson et al., 1996).

The apparent potential to polarize the immune character by simply altering the gene sequence encoding the antigen, such that expression generated either a cell-associated or secreted form of the antigen, is critical for vaccine design. Surprisingly, immunization of C57BL/6 (H-2^b) mice with the plasma membrane anchored form of BHV-1 gD did not result in a predominance of serum IgG_{2a}. While there was an increase in the levels of IgG_{2a} in these mice, as compared to animals receiving plasmid encoding the secreted form of gD, this outcome was surprising given that this strain of mouse, like C3H/HeN, show a similar Th1 type response to *Leishmania* infection (Locksley and Scott, 1991). While it has been demonstrated that C3H/HeN mice tend to overproduce IFN- γ in response to certain antigens, it does suggest the important influence mouse strain can have on immune responses to a given antigen (Major and Cuff, 1996). Based on serum IgG subclass profiles in C57BL/6 mice immunized with plasmids encoding secreted and membrane anchored BHV-1 gD, one might predict that the Th2 predisposed Balb/c (H-2^d) mice would show an exclusively IgG₁ response to all of these antigens (Major and Cuff, 1996). However, several papers published recently have demonstrated that Balb/c mice are capable of systemic IgG_{2a} predominance following immunization with a variety of DNA-based vaccines (Manickan et al., 1995; Pertmer et al., 1996; Raz et al., 1996). In light of these data, the danger of making claims describing the universality of the immune responses to DNA-based vaccines is premature. It is also important to recognize that vaccine testing in different mouse strains, showing genetic predispositions (H-2^d linkage to IgG₁) towards development of a particular immune outcome, represents an opportunity to emulate responses in outbred target populations.

There are three other factors which can influence the type of immune response that occurs following immunization of DNA-based vaccines: intrinsic, immunologically unique features of encoded antigens, route and method of immunization, and the development of CTL responses (Huygen et al., 1996; Mancini et al., 1996; Pertmer et al., 1996; Feltquate et al., 1997). Immunization of C57BL/6 mice i.m. with a plasmid encoding the secreted antigen Ag85 from *M. tuberculosis*, resulted in a predominance of serum IgG_{2b} and IgG_{2a} (Huygen et al., 1996). However, immunization of C57BL/6 mice with plasmids encoding the Hepatitis B surface antigen results in secretion of virus like particles (VLPs) and in the predominance of IgG_{2a} (Mancini et al., 1996). Similarly, the secreted form of BHV-1 gD elicited an almost exclusive production of IgG₁ in these H-2^b mice. Cardoso et al. (1996), demonstrated that i.m. immunization of Balb/c mice with plasmids encoding either cell membrane anchored, or partially secreted, forms of Measles virus hemagglutinin elicited a predominance of serum IgG_{2a} and IgG₁, respectively (Cardoso et al., 1996). Conversely, a plasmid encoding a secreted form of the amino-terminal portion of Hepatitis C virus nucleocapsid failed to deviate the serum IgG isotype towards greater levels of IgG₁ following i.m. injection into the tibialis anterior muscles of Balb/c mice (Inchauspe et al., 1997). Despite this apparently controversial data, it is evident that soluble antigen is handled in a manner unique from that occurring with cell-associated antigen. For example, administration of soluble aqueous antigen has been demonstrated to either tolerize both Th subsets or, in some cases, to selectively tolerize Th1-type cells (Burstein et al., 1992; Chu et al., 1995). Subcutaneous immunization with mini-osmotic pumps designed to release a chronic low

dose of soluble antigen has been demonstrated to selectively expand antigen-specific Th2 responses (Guery et al., 1996). The mechanism for this outcome is unknown. However, it was hypothesized that targeting a specific antigen presenting population may be responsible for the observed immune character. Indeed B-cells of splenic origin have been demonstrated to selectively expand Th2 clones (Gajewski et al., 1991) and to preferentially activate interleukin 4 production (Rossi-Bergman et al., 1993). It has also been demonstrated that dendritic cells from different tissues can be functionally distinct and induce profound polarization of T-cell phenotypes (Hill et al., 1994; Everson et al., 1996). In short, a clear mechanistic understanding for the observed differences in immune character to soluble and cell-associated antigens is not immediately forthcoming. However, our preliminary data suggests that DNA-based vaccine delivery of antigens engineered to be expressed within, or outside of, host cells may offer a mechanism whereby the immune response can be manipulated to some extent.

The route, or rather the method of immunization, has also been demonstrated to have a major impact on the character of the ensuing immune repertoire (Pertmer et al., 1996; Feltquate et al., 1997). Gene gun administration of DNA-based vaccines tends to result in a predominance of serum IgG₁. However, it has been demonstrated that ballistic immunization of C3H/HeN mice with plasmid encoding the influenza nucleoprotein resulted in IgG_{2a} as the predominant serum isotype (Pertmer et al., 1996). While it does seem that ballistic and i.m. delivery of DNA-based vaccines represent opposite ends of the humoral isotypic spectrum it is not, perhaps, surprising when one considers the vast difference in immunological microenvironments these two anatomical sites represent

(Bos and Kapsenberg, 1993; Hohlfeld and Engel, 1994). Furthermore, the importance of the specific IgG isotype may be of questionable protective value, particularly if high neutralizing titers of IgG are present in the sera of animals (Bachmann et al., 1997). However, given that most protective immune responses to viral infections in mice are characterized by a predominance in IgG_{2a}, and that this isotype is much more efficient at complement fixation, it remains an issue of some importance in vaccine strategy (Klaus et al., 1979; Coutelier et al., 1988; Coutelier et al., 1991; Nguyen et al., 1994).

Although we have not been able to demonstrate BHV-1 gD specific CD8⁺ mediated cytolytic activity in several strains of mice, the importance of this component of the immune repertoire in controlling many noncytopathic viral, and possibly cytopathic, diseases must not be understated (Ramsay et al., 1993; Bloom and Zinkernagel, 1996; Zinkernagel, 1996). Generally speaking, DNA-based vaccines, regardless of the route of vaccine administration, type of antigen, or strain of mouse, are highly effective in generating a potent CTL population against encoded antigens (Armas et al., 1996; Ciernik et al., 1996; Tascon et al., 1996). We have demonstrated that immunization of mice with plasmid encoding authentic as well as deletion mutants of BHV-1 gD results in highly efficacious humoral and cell-mediated immunity. We have also demonstrated that i.m. immunization of C3H/HeN mice with cell-associated forms of BHV-1 gD antigen leads to a splenic, lymph node and serum antibody isotype profile consistent with a Th1-like immune phenotype. However, mice immunized with a secreted form of this same antigen display immune responses characterized by greater levels of the IL-4 in the draining lymph node and IgG₁ as the predominant serum isotype.

DNA-based vaccines represent a novel, simple method to achieve potent immune responses that include humoral, and cell-mediated immunity. Clearly the potential for these vaccines to induce potent Th1-type immune responses, often in conjunction with high levels of IgG_{2a} and cytolytic activity, necessitates further exploration. We have also reached the point where modulation of these immune responses are being investigated such that the appropriateness, magnitude, duration, and balance of resulting repertoires emulate "normal" protective immune responses observed in convalescent animals. In light of the demonstration that cell association of plasmid delivered antigens has a significant impact on the developing immunological phenotype, we might consider utilizing several different antigens from the same pathogen as the basis for generating a balanced, comprehensive immune response (Doolan et al., 1996). Further, the evidence that Th1 and Th2 (as well as IgG_{2a} and IgG₁) responses are not mutually exclusive within the same animal, and that early compartmentalization of B-cell and T-cell responses may allow the propagation of this apparent immunological paradigm, suggests that multivalent vaccines encoding both cell surface (ie. viral envelope glycoprotein) and intracellular (viral intracellular structural and nonstructural proteins) nonstructural proteins may be a more logical mode of generating the appropriate protective immune response. Whether this approach will require anatomically separate dosing to avoid bystander modulation effects remains to be seen (Sangster et al., 1997). Finally, research with HIV has led to the development of the hypothesis that strong CMI or humoral immunity may be mutually exclusive within the same animal and that forced maintenance of both may be immunologically deleterious to the individual in which they occur (Shearer and Clerici,

1997). Ultimately, the potential for many DNA-based vaccines to elicit both cell-mediated and humoral immunity, in conjunction with longevity of these responses, will test theories regarding self regulation of each, or both, arms of the immune repertoire.

4.0 IMMUNE AUGMENTATION AND DEVIATION IN MICE IMMUNIZED WITH PLASMIDS ENCODING EIGHT CYTOKINES CO-ADMINISTERED WITH A DNA-BASED VACCINE ENCODING A SOLUBLE FORM OF BOVINE HERPESVIRUS-1 GLYCOPROTEIN D

4.1 Introduction

Immunization with naked DNA, variously described as genetic, polynucleotide, nucleic acid or DNA-based immunization, involves the *in vivo* delivery of, typically, plasmid DNA comprised of gene expression regulatory elements and gene(s) encoding antigen (Tang et al., 1992; Hassett and Lindsay Whitton, 1996). Immunogens derived from viral, bacterial, and parasitic pathogens, as well as, reporter genes, genes encoding tumour rejection antigens, and those involved in modulation of autoimmune disorders, have been introduced *in vivo* as plasmid encoded constructs and many have resulted in significant immune responses including, in appropriate animals models, protection following challenge (Donnelly et al., 1993; Barry et al., 1995; Waine and McManus, 1995; Anderson et al., 1996; Conry et al., 1996; Doolan et al., 1996; Haynes et al., 1996; Hsu et al., 1996; Irvine et al., 1996; Raz et al., 1996; Syrengelas et al., 1996; Tascon et al., 1996; Waisman et al., 1996; Whalen, 1996; Ulmer et al., 1996a; d'Oliveira et al., 1997; Luke et al., 1997; McDaniel et al., 1997). Routes and methods of delivery of plasmids encoding reporter genes and a variety of protective immunogens includes:

topical, unfacilitated delivery, (mucosal) (Fynan et al., 1993) injection (intramuscular, intradermal, intraperitoneal and intravenous) (Wolff et al., 1992; Robinson et al., 1993; Zhu et al., 1993; Raz et al., 1994), ballistic (primarily dermal and epidermal)(Haynes et al., 1996), liposomal (Li and Hoffman, 1995; Wheeler et al., 1996), modified live viral (Yoshimura et al., 1993), bacterially facilitated delivery (epidermis and mucosa)(Sizemore et al., 1995), and transfected cell delivery (Ulmer et al., 1996c). Many of these delivery mechanisms have resulted in demonstrable numbers of transfected cells *in vivo*, and most have resulted in antigen-specific immune responses representing both humoral and cell-mediated arms of immunity (Wolff et al., 1992; Condon et al., 1996; Norman et al., 1997). Despite initial successes with this novel technology, researchers studying immune responses to DNA-based vaccines are now discovering that not all antigens are created, or perceived, equally (Mougneau et al., 1995; Hermann et al., 1996; Milich et al., 1997; van Drunen Littel-van den Hurk et al., 1998). Indeed, there is ample evidence that the intrinsic antigenic qualities associated with specific immunogens may deviate or modulate immune responses in a variety of ways (Cardoso et al., 1996; Spriggs, 1996). We have demonstrated, using DNA-based vaccines, in mice and cattle that the cellular compartment in which the same antigen is expressed can significantly alter the immune outcome following immunization (Lewis et al., 1997; van Drunen Littel-van den Hurk et al., 1998). Despite immunogenic features intrinsic to individual antigens, it seems apparent that many, but not all, immune responses, following i.m. or i.d. delivery of DNA-based vaccines in mice are characteristic of a Th1-type immune response and typically includes a potent CD8⁺ CTL response (Ertl and Xiang, 1996;

Ulmer et al., 1996b). Th1-type phenotypes are characterized by a predominance of antigen-specific IL-2, IFN γ and lymphotoxin secreting CD4⁺ T-cells, potent DTH, and often a predominance of serum IgG₂ (Mosmann et al., 1986; Snapper and Paul, 1987). Th2-type phenotypes are characterized by CD4⁺ T-cells that secrete IL-4, 5, 6, 10 and 13 and often display elevated serum IgE or a predominance of IgG₁ (Mosmann et al., 1986; Snapper and Paul, 1987). What is becoming increasingly apparent is that intrinsic antigenicity, microenvironmental locale of the antigen, route or method of DNA delivery, as well as dose of the vaccine all influence the magnitude and character of the ensuing immune response (Pertmer et al., 1996; Coffman and von der Weid, 1997; Feltquate et al., 1997; Lewis et al., 1997; Roman et al., 1997).

Cellular and molecular events at the site of vaccination, in transit from the site of vaccination, within the various subcompartments of lymphoid organs draining the vaccination site, and ultimately redistribution of mediators of humoral and cell-mediated immunity are orchestrated to a large extent by cytokines and chemokines (Hughes and Babiuk, 1992; del Pozo et al., 1996; Lofthouse et al., 1996; Premack and Schall, 1996). While a wide array of recombinant cytokines and chemokines are available for humans and mice, this has not been the case for many species of interest in veterinary medicine. Also, research involving use of conventionally prepared recombinant cytokines is often prohibited by large, occasionally toxic doses required, and by exorbitant costs of production (Heath and Playfair, 1990; Hughes and Babiuk, 1992; Finkelman and Maliszewski, 1997). DNA-based vaccine technology has provided justification and impetus to clone new cytokines and chemokines from both food and companion animals

of veterinary interest. The impetus to do so is on the understanding that costs of protein purification and administration would be greatly reduced because injection grade expression plasmids encoding cytokines are easily created and purified and may be administered prophylactically in the context of conventional, or DNA-based vaccines. A relatively cost effective system for prescreening of cytokines, or chemokines, sets the stage for rapid and efficient identification of adjuvant potential, and for the immunological or molecular dissection of the mechanisms of immune response. A variety of recombinant cytokines including; IL-1 α and β , IL-2, IL-4, IL-6, IL-7, GM-CSF, IFN γ , TNF α , TGF β and IL-12 have been utilized to modulate immune responses to specific antigens (Dranoff et al., 1993; Raz et al., 1993; Taylor, 1995; Irvine et al., 1996; Lofthouse et al., 1996; Rao et al., 1996). We chose to investigate the potential immunomodulatory effects of plasmids encoding IL-1 α , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN γ and TNF α when co-administered i.m. with plasmids encoding a viral antigen.

Interleukin (IL)-1 α and (IL)-1 β display many similarities in biological function and have both been demonstrated to enhance antigen-specific immune responses (Dinarello, 1991). These cytokines are secreted from a variety of lymphoid and nonlymphoid cells including: most professional antigen presenting cells, epithelial cells, keratinocytes, as well as, B- and T-cells. The specific immunological role of these cytokines are unclear, however, they have been demonstrated, *in vitro*, to play a role in B-cell differentiation, antibody secretion and induction of IL-2. Certainly, the early, post insult, appearance of these cytokines suggests a pivotal role in the development of innate and cognate

immunological responses that follow the early stages of infection. Interleukin (IL)-2 is secreted by activated Th0 and Th1 cells, and is instrumental in the induction of CTL activity, IFN γ production, and B-cell stimulation (Weinberg and Merigan, 1988). Administration of this cytokine at the time of immunization has been observed to augment protective immunity to several viral and parasitic vaccines (Lofthouse et al., 1996). Interleukin (IL)-4 is secreted by a variety of cells, including cells of the Th0 and Th2 phenotype, as well as CD4⁺, NK 1.1⁺ T-cells (Mosmann et al., 1986; Street et al., 1990; MacDonald, 1995). IL-4 has been demonstrated to block IL-2 dependent proliferation of T-cells and promote the differentiation of T_{CTL} into noncytolytic T_{C2} IL-4 secreting phenotype, and also appears to indirectly inhibit IL-12 and IFN γ mediated immune deviation towards a Th1-type phenotype (Powrie and Coffman, 1993; Croft et al., 1994; Seder and Le Gros, 1995; Rocken et al., 1996). IL-4 has also been shown to influence the switching of B-cell immunoglobulins to the IgG₁ and IgE isotypes and to be involved in maintenance of dendritic cell antigen processing capabilities (Snapper and Paul, 1987; Sallusto and Lanzavecchia, 1994). In this way IL-4 is an inhibitor of Th1 responses and a promoter of Th2 responses to antigens (Dranoff et al., 1993; Sharma et al., 1996; Flesch et al., 1997). Interleukin (IL)-6 is a multifunctional cytokine produced by a number of lymphoid (macrophages and activated CD4⁺ T-cells of the Th2 phenotype) and nonlymphoid (keratinocytes, fibroblasts, endothelial) cells, (Hirano et al., 1990). This cytokine is very pleiotropic in function and has been demonstrated to induce terminal B-cell differentiation and to enhance the production of IgA at mucosal surfaces (Ramsay et al., 1994). Furthermore, creation of attenuated recombinant Vaccinia

or Fowlpox viruses that express IL-6 can enhance primary and recall humoral antibody responses in mice (Leong et al., 1994). Interleukin (IL)-10 is secreted primarily by Th2-type T-cells and inhibits Th1-type immune responses possibly through downregulation of macrophage and possibly dendritic accessory cell functions (Fiorentino et al., 1991; De Smedt et al., 1997). Tumour necrosis factor (TNF)- α is secreted primarily by monocytes and macrophages and induces hemorrhagic necrosis of tumours and initiates a metabolic cascade leading to systemic shock associated with severe disease conditions (Manogue et al., 1991). TNF α also shares some biological properties with IL-1 and has been demonstrated to play a role in onset, and augmentation, of immune responses to pathogens or vaccines (Durum et al., 1985; Nathan, 1987). Recently TNF α has been demonstrated to play an important role in the maturation, and perhaps mobilization of nonlymphoid tissue resident dendritic cells (Sallusto and Lanzavecchia, 1994). *In vivo* work in cattle showed an increase in peripheral blood monocyte numbers and function after administration of recombinant, E. coli derived, bovine TNF α , (Bielefeldt-Ohmann et al., 1989). This cytokine has also been demonstrated to specifically enhance *in vivo* immune responses to a T-dependent antigen in mice (Ghiara et al., 1987). Interferon (IFN)- γ is produced by activated Th0 and Th1-type CD4⁺ T-cells and by IL-12 (or IL-1) activated NK cells (Mosmann and Coffman, 1987; Street et al., 1990; Scharon and Scott, 1993). It has been primarily characterized in terms of its ability to upregulate MHC class I and II expression on antigen presenting cells (Rosa and Fellous, 1984; Mosmann and Coffman, 1989). More recently this cytokine has been shown to regulate, in part, deviation of the immune response towards a Th1 phenotype and as an immunoglobulin

isotype switching factor involved in the generation of IgG_{2a} by T-independent and T-dependent B-cell responses (Snapper and Mond, 1993). It also appears that this cytokine is more effective in deviating immunity towards a Th1-type of response when synergized with IL-12 (Schijns et al., 1995; Ahlers et al., 1997). There is also direct evidence in mice that co-administration of recombinant murine IFN γ with conventional vaccines, results in enhanced humoral responses (Lofthouse et al., 1996). Finally, granulocyte/macrophage-colony stimulating factor (GM-CSF) acts on bipotential bone marrow stem cells to produce mononuclear phagocytes, including dendritic cells, and granulocytes (Morrissey et al., 1987; Fischer et al., 1988). GM-CSF has also been demonstrated to recruit and enhance the activation of professional antigen presenting cells (APCs), to upregulate major histocompatibility antigen II (MHC II) on APCs and augment the primary antibody response following vaccination (Heufler et al., 1987; Fischer et al., 1988; Elliot et al., 1991; Inaba et al., 1992).

A number of recent publications have characterized immune responses to several antigens, delivered as protein or as DNA-based vaccines, following co-delivery of plasmids encoding a variety of cytokines. Cytokines tested to date include: IL-2, IL-4, IL-6, IL-7, IL-12, GM-CSF and TGF β (Raz et al., 1993; Xiang and Ertl, 1995; Irvine et al., 1996; Chow et al., 1997; Tsuji et al., 1997). Prior to the commencement of experiments described in this section, no single published report described immune responses to plasmids encoding cytokines co-administered with DNA-based vaccines. We chose to investigate 8 different murine cytokines based on historical data describing either adjuvant or specific APC modulatory capacity. Interleukins 1 α , 2, 4, 6, 10, as well as,

TNF α , IFN γ and GM-CSF, were subcloned into an expression cassette and co-administered with a second plasmid encoding a secreted version of bovine herpesvirus-1 (BHV-1) glycoprotein D (SgD). Authentic glycoprotein D normally displays a transmembrane domain that anchors it within the host cell plasma membrane, and subsequently on the viral lipid envelope (Tikoo et al., 1990). This Alphaherpesvirus is of significant economic importance in the cattle industry due to its involvement in a variety of clinical and subclinical diseases conditions. It is the causative agent of Infectious Bovine Rhinotracheitis (IBR) and infectious pustular vulvovaginitis and balanoposthitis (IPV and IPB respectively) in cattle (Roizman et al., 1981; van Drunen Littel-van den Hurk et al., 1993a). BHV-1 is also believed to play a significant role in abortions, meningoencephalitis and conjunctival diseases (Schuh, 1994). Finally, BHV-1 is considered to be the single most important agent predisposing cattle to secondary, opportunistic, bacterial infections leading to a bovine respiratory disease complex commonly referred to as Shipping Fever (Yates, 1982; Babiuk et al., 1996).

Protective immune responses are elicited in cattle immunized with BHV-1 gD as determined by reduced shedding of virus and a significant reduction in clinical signs and susceptibility to secondary bacterial infection (van Drunen Littel-van den Hurk et al., 1993b). Immunity to this antigen in mice, both as a conventional vaccine and as a DNA-based vaccine, has also been studied extensively (Cox et al., 1993; Baca-Estrada et al., 1996; Braun et al., 1997; Lewis et al., 1997; van Drunen Littel-van den Hurk et al., 1998). Although it is not possible to carry out viral challenges, in mice, because of the inability of this species to support a full replication cycle of BHV-1, we feel that preliminary

screening of cytokine modulatory capabilities in this species is justified. Criteria for the determination of modulatory capacity of co-administered cytokines included: impact on serum antigen-specific serum antibody levels, ratio of serum IgG₁ and IgG_{2a}, and the impact on seroconversion efficacy. We included T- and B-cell ELISPOT data from cell populations harvested from spleens and draining lymph nodes to further understand what factors might be contributing to observed deviations or augmentations in the immune responses observed. We were able to demonstrate that augmentation of humoral responses, as well as, seroconversion efficiency, occurred if a single low dose of plasmids encoding GM-CSF, IL-4, IL-6 and TNF α were co-administered with a suboptimal dose of plasmids encoding a secreted form of BHV-1 gD. Enhancement of the serum levels of IgG_{2a} occurred when plasmids encoding IFN γ and TNF α were co-administered with plasmids encoding a secreted form of BHV-1 gD. Finally, dose titration studies with plasmids encoding GM-CSF and IFN γ showed unequivocal evidence that dose of plasmid encoded cytokine and the presence or absence of boosting can have dramatic effects on the character of the ensuing immune response.

4.2 Materials and Methods

4.2.1 Mouse Strain

Female 5-6 week old C3H/HeN mice were purchased from Charles River (Canada). All mice were acclimated to new housing for 1-2 weeks prior to vaccination with DNA-based or conventional vaccines. All mice were immunized with DNA-based

vaccines in the left and right quadriceps muscle masses with or without boosts 2 weeks later.

4.2.2 Bacterial Strains, Mammalian Cell Lines and Tissue Culture Reagents.

Bacterial *E. coli* cells included: JM105 (New England Biolabs, Inc., Mississauga, Ont.), HB101 (Invitrogen Corp., San Diego, CA), DH 5 α (CLONTECH Lab., Inc., Palo Alto, CA), and SURE[®] cells (Stratagene, La Jolla, CA). Murine cell lines included: L929 (ATCC No. CCL 1); D10.G4.1 (ATCC No. TIB 224); HT-2 (ATCC No. CRL 1841); CTLL-2 (ATCC No. TIB 214); 7TD1 (ATCC No. CRL 1851); COS-7 (ATCC No. CRL 1651); P388D1 (ATCC No. TIB-63); WEHI-3 (ATCC No. TIB-68) and the GM-CSF sensitive cell line DA-1k (T. Mossman, DNAX Corp., 1986). LM-1 is a subline of D10.G4.1 developed in our lab (L. McDougall, Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada) that does not require spleen feeder cells or conalbumin. LM-1 cell growth requires only the presence of 10% rat growth factor. All media and media supplements were purchased from GIBCO-BRL (Gibco, Life Technologies, Burlington, Ont.) or Sigma (Sigma Chemical Co., St. Louis, MO) unless otherwise indicated. All tissue culture plasticware was purchased from Corning (Corning Inc., Corning, N.Y.) or Costar (Costar/Nucleopore Canada Inc., Toronto, Ont.) unless indicated otherwise.

4.2.3 Plasmid DNA and Molecular Reagents

Commercial plasmids included pSL301 and pCDNA3 (Invitrogen), and pGEM1

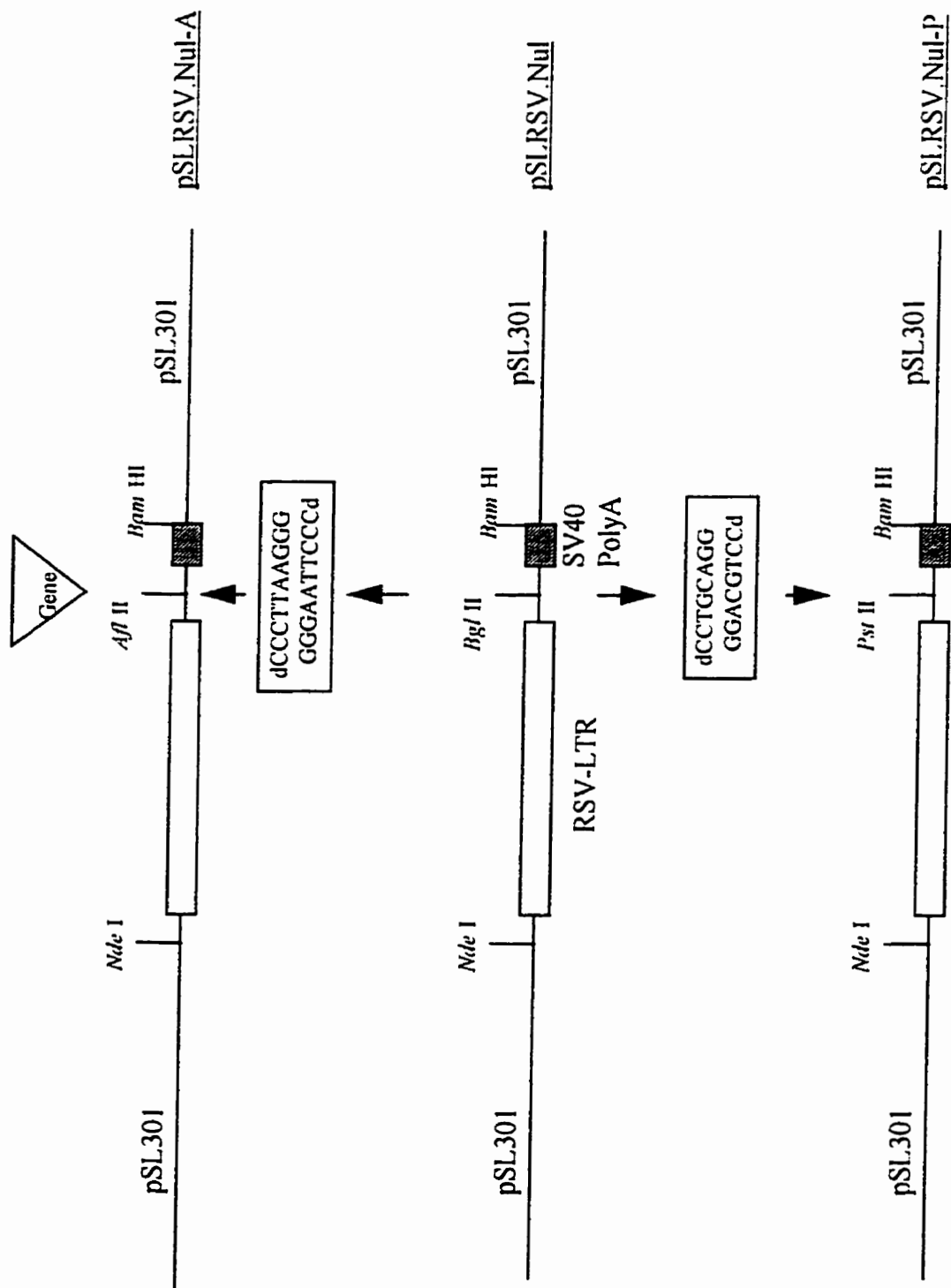
(Promega Corp., Madison, WI). The construction of expression cassette pSLRSV.Nul has been described previously (section 3.2.1). Two additional expression cassettes were created based on pSLRSV.Nul, that involved ablation of the unique *Bgl* II site within this plasmid and insertion of an *Afl* II or *Pst* I site (Fig. 4.1). An *Afl* II site was inserted by blunt- end ligation of an *Afl* II linker (dCGCTTAAGCG-New England Biolabs, Mississauga, Ont.) or *Pst* I linker (dCCTGCAGG). The DNA-based vaccine expressing a secreted form of glycoprotein D from BHV-1 has been described previously (section 3.2.1). All restriction endonucleases and DNA/RNA modifying enzymes were from Pharmacia (Pharmacia Biotech., Quebec) or New England Biolabs. All purifications of mRNA and first strand cDNA synthesis was carried out using kits purchased from Pharmacia. All injection quality plasmid DNA was prepared by anion-exchange column purification (Qiagen, Inc., Santa Clarita, CA) and assessed by restriction analysis and agarose gel electrophoresis prior to injection.

4.2.4 Construction of plasmids expressing murine cytokine genes

All eight cytokine cDNAs (IL-1 α , IL-2, IL-4, IL-6, IL-10, GM-CSF, TNF α and IFN γ) were inserted into the expression cassette pSLRSV.Nul which had been modified to receive *Pst* I (pSLRSV.Nul-P) or *Afl* II (pSLRSV.Nul-A) restricted genes (Fig. 4.1). Murine cDNAs encoding IL-2 (1170 bp) and GM-CSF (940) in pGEM1[®] (Promega) vector was kindly provided by Dr. J. Elliot (Dept. Medical Microbiology and Infectious Diseases, University of Alberta, Canada).

Plasmid pSLRSV.IL-2 was generated by first ligating an *Afl* II linker sequence (New

Fig. 4.1 Schematic diagram of the eukaryotic expression cassettes. Gene expression modulatory elements are on a pSL301[™] plasmid backbone and are comprised of the promoter/enhancer from Rous sarcoma virus long terminal repeat and the polyadenylation signal from SV40. Creation of a plasmid with unique *Pst* I (pSLRSV.NUL-P) or *Afl* II (pSLRSV.NUL-A) gene insertion sites was carried out by digestion of the parent cassette (pSLRSV.NUL-B), containing a unique *Bgl* II insertion site, with *Bgl* II followed by end-repair and ligation of either an *Afl* II or *Pst* I linker sequence. Murine cytokine cDNAs were inserted in these unique restriction sites depending on the presence or absence of these sites in the sequence of the particular cytokine. Murine cytokines: IL-1 α , IL-10, GM-CSF and IFN γ were inserted into the pSLRSV.NUL-B vector. Murine cytokines IL-2 and IL-6 were inserted into the pSLRSV.NUL-A vector and murine cytokine TNF α was inserted into the pSLRSV.Nul-P vector.



England Biolabs) into the unique *Pst* I site within the 5'-untranslated (UT) region, by *Pst* I digestion followed by Klenow end-repair and blunt-end linker ligation. Excision of the IL-2 gene from pGEM1 was carried out by *Afl* II - *Ssp* I (3'-UT region) digestion and ligation into *Afl* II - *Eco*47 III digested pSL301 (Invitrogen). A positive clone was digested with *Eco* RV and the *Afl* II linker ligated into this site. Transformation and identification of IL-2 with *Afl* II unique restriction sites within the 5' and 3'-UT regions allowed excision and ligation into the unique *Afl* II restriction site within the expression cassette pSLRSV.Nul-A to create the plasmid pSLRSV.IL-2.

Plasmid pSLRSV.CSF was created by digesting the pGEM vector containing this cDNA with *Sau* I and *Tfi* I. For convenience we chose to utilize the second initiation codon within the translated region of the gene. It has been demonstrated that this second ATG start site is utilized to the same extent as the initial initiation codon and that the resulting product maintains biological activity equal to its longer counterpart (Gough et al., 1985). *Bgl* II linkers were ligated to the end-repaired *Sau* I/*Tfi* I fragment. After ligation the DNA fragment was predigested with *Bgl* II, heat inactivated, and purified following agarose gel electrophoresis using a GeneClean[™] (BIO 101, Inc., Vista, CA) kit. The GM-CSF cDNA with *Bgl* II ends was ligated into vector pSLRSV.Nul-B to generate pSLRSV.GM-CSF.

Plasmid pSLRSV.IL-4 was created by amplifying the IL-4 first strand cDNA using polymerase chain reaction (PCR) from reversed transcribed messenger RNA (mRNA) harvested from C57BL/6 immune splenocytes harvested 3 weeks post-boost, and stimulated with antigen (150 ng/mL at 10 mL/25 million splenocytes) for 48 hours *in*

vitro. Sense and antisense primers (Table 4.1) commenced at bases 41 and 506, respectively, of the published IL-4 cDNA sequence (Lee et al., 1986). These primers introduced unique *Bgl* II restriction sites within the 5' and 3' untranslated regions. The amplified IL-4 sequence was phosphorylated with T4 polynucleotide kinase, phenol:chloroform extracted, and purified using a Geneclean™ kit. This 465 bp cDNA was blunt-end ligated into *Eco* RV cut pCDNA3[®] (Invitrogen) and used to transform competent DH 5 α cells. Positive clones were identified and used to provide an IL-4 cDNA with *Bgl* II ends that was subsequently ligated into expression cassette pSLRSV.Nul to create plasmid pSLRSV.IL-4.

Plasmid pSLRSV.IL-6 was created by amplifying reverse transcribed mRNA prepared from splenocytes harvested from C3H/HeN mice immunized with a DNA-based vaccine (pSLRSV.SgD) and boosted two times with recombinant, affinity column purified, truncated gD (tgD) formulated with VSA3 (Biostar Inc., Saskatoon, Saskatchewan, Canada). Sense and antisense primers (Table 4.1) commenced at bases 15 and 671, respectively, of the published cDNA sequence (Van Snick et al., 1988). These primers also introduce unique *Afl* II restriction sites into the 5' and 3'-UT regions. Amplified IL-6 cDNA was phosphorylated and ligated into *Eco* RV digested, dephosphorylated vector, pSL301. Following transformation of competent DH 5 α bacterial cells, positive clones were identified and the IL-6 cDNA, with unique flanking *Afl* II ends, was subcloned into the *Afl* II site of pSLRSV.Nul-A to create plasmid pSLRSV.IL-6.

Plasmid pSLRSV.IL-10 was created by amplifying, by PCR, reverse transcribed

Table 4.1 Primer sequences utilized to generate PCR amplified cytokine cDNAs.

Cytokines		Primer Sequences		Restriction Sites
				5' and 3' ends
IL-1 α	S	5'-CTGTCAGATCTTCCCAAGT GAATAGAC-3'		Bgl II
	A	5'-GCAAAGATCTCAAGATGGCCAAAGTTCC-3'		
IL-4	S	5'-CGCACAGATCTATTGATGGGTCTCAACCCCCAG-3'		Bgl II
	A	5'-CATAAGATCTAGCATGGTGGCTCAGTACTACGAG-3'		
IL-6	S	5'-GTCAACTTAAGAAACCGCTATGAAGTTCC-3'		Afl II
	A	5'-TGCTTAAGCATAACGCACTAGGTTTGC-3'		
IL-10	S	5'-AGAGATCTCCATCATGCCTGGCTCAGC-3'		Bgl II
	A	5'-GCAGATCTTTTAGCTTTTCATTTTG-3'		
TNF α	S	5'-GCCCAAGGCGCCACATCTCCCTGCAGAAA AG-3'		Pst I
	A	5'-AATGGCTGCAGACCCATTCCCTTCACAGAG-3'		
IFN γ	S	5'-CTAGATCTGAGACAATGAACGCACACT-3'		Bgl II
	A	5'-CCAGATCTTCCCCACCCCGAA TCAGC-3'		

S - sense strand
A - Antisense strand

S - sense strand

A - Antisense strand

mRNA prepared from the murine helper T-cell line D10.G4.1. A subclone of this line (LM-1), was grown in Click's media (GIBCO-BRL) with 50 μ m 2-mercaptoethanol, 10% rat growth factor containing 20 mg/ml α -methyl-D-mannoside and 10% fetal bovine serum (FBS). LM-1 cells were stimulated with conalbumin A, at 4 μ g/mL, for 24 hours prior to extraction of total RNA. Sense and antisense primers (Table 4.1) commenced at bases 61 and 619, respectively, of the published sequence (Moore et al., 1990). Primers

also introduced unique *Bgl* II sites into the 5' and 3'-UT regions. PCR products were purified, following agarose gel electrophoresis, and phosphorylated prior to blunt-end ligation into *Eco* RV digested, dephosphorylated, pSL301. Following transformation of competent SURE[®] cells, positive clones were identified and anion-exchange column purified plasmid DNA from a single positive clone was digested with *Bgl* II to generate an IL-10 cDNA fragment with *Bgl* II sticky ends. This fragment was ligated into the *Bgl* II site of pSLRSV.Nul to generate plasmid pSLRSV.IL-10.

Plasmid pSLRSV.TNF α was generated by amplifying reverse transcribed total RNA from the macrophage cell line P388D1. P388D1 cells were stimulated with LPS (10 μ g/mL) for 2 and 5 hours and total RNA extracted and pooled. Following first strand cDNA synthesis, sense and antisense primers (Table 4.1) were utilized to generate a 765 bp PCR product that commenced at bases 107 and 872, respectively, of the published sequence (Pennica et al., 1985). These primers introduced unique *Pst* I sites into the 5' and 3'-UT region of the cDNA. This PCR product was digested with *Pst* I prior to ligation into dephosphorylated, *Pst* I digested, pSL301. Positive clones were identified and anion-exchange column purified plasmid DNA, from a single positive clone, was digested with *Pst* I to provide TNF α cDNA. Following agarose gel electrophoresis and purification, this cDNA was ligated into *Pst* I digested expression cassette pSLRSV.Nul-P to generate plasmid pSLRSV.TNF α .

Plasmid pSLRSV.IFN γ was created by PCR facilitated amplification of reverse transcribed mRNA from immune murine spleens. C57BL/6 mice were immunized with recombinant truncated gD (500 ng/mouse) formulated in VSA3. Mice were boosted at 3

weeks and spleens harvested 3 weeks following boost. Splenocytes were stimulated 48 hours *in vitro*, with truncated gD (150 ng/tgD/mL and 10 mL/25 million splenocytes). Sense and antisense PCR primers (Table 4.1) commenced at bases 55 and 558 , respectively, of the published sequence (Gray and Goeddel, 1983). These primers introduce unique *Bgl* II sites within the 5' and 3'-UT regions. The 503 base pair, purified PCR product, was phosphorylated and blunt-end ligated into *Eco* RV digested, dephosphorylated, pSL301. Positive clones were identified and anion-exchange column, purified plasmid DNA from a single clone was digested with *Bgl* II and the IFN γ cDNA ligated into the expression cassette pSLRSV.Nul to create the plasmid pSLRSV.IFN γ .

Plasmid pSLRSV.IL-1 α was created by PCR amplification of reverse transcribed mRNA prepared from LPS stimulated P388D1 cells. Sense and antisense primers commenced at bases 46 and 919 of the published sequence and generate an 873 bp product (Lomedico et al., 1984). These primers introduce unique *Bgl* II restriction sites into the 5' and 3'-UT regions. Following agarose gel electrophoresis and purification, the PCR product was digested with *Bgl* II purified once again and ligated into *Bgl* II digested, dephosphorylated, expression cassette pSLRSV.Nul. All polymerase chain reactions were carried out using VentTM DNA polymerase (New England Biolabs), 32 cycles of amplification on a Model PTC-1 (GIBCO BRL, Inc.) thermocycler. Restriction pattern analysis (all cytokine cDNAs), partial dideoxy sequencing (IL-6, IL-10, TNF α and IFN γ) and functional bioassays (all cytokine expression constructs) were utilized to confirm identity of cytokine cDNAs utilized in this research.

4.2.5 Transient transfection of COS-7 cells with cytokine expression cassettes.

COS-7 cells, at 70-80% confluency, in six well tissue culture plates were washed twice with Hanks Balanced Salt Solution (GIBCO/BRL). Expression cassettes encoding eight different murine cytokine cDNAs were purified by anion-exchange column chromatography. Two μg of each plasmid, as well as a control pSLRSV.Nul plasmid was diluted into 500 μL serum free Dulbecco's Modified Eagle Medium (DMEM) (GIBCO/BRL). Transfectam[®] (Promega), (5.0 μL of 2.5 $\mu\text{g}/\mu\text{L}$ stock) was diluted in a separate 500 μL volume of DMEM and vortexed briefly. DMEM containing plasmid DNA and Transfectam[®] were mixed and immediately added to washed COS-7 monolayers. Monolayers were incubated for 5 hours with the Transfectam[®]/DNA mixture at which point 1.5 mLs of DMEM containing 20% FBS (GIBCO-BRL) was added to each transfection well. Media from each transfection were harvested after 24 hours and centrifuged (30 seconds at 16,000 x g) briefly to remove cellular debris. Cleared media was transferred to clean sterile 1.5 mL Eppendorf tubes and stored at -20 °C until needed for cytokine bioassays.

4.2.6 Cytokine bioassays

Commercially prepared recombinant, human IL-6, murine IL-10, IFN γ , and GM-CSF were purchased from Genzyme Corp. (Cambridge, MA)), recombinant murine IL-4 was purchased from Pharmingen (San Diego, CA), recombinant murine IL-1 β and TNF α were purchased from Boehringer Mannheim Canada Ltd. (Laval, Quebec), and recombinant murine IL-2 was purchased from ICN Biomedicals Canada Ltd. (St-Laurent,

Quebec). Cytokine bioassays using media from COS-7 cells transiently transfected with each of the cytokine expression constructs (excluding pSLRSV.IL-10) were carried out according to described protocols (Coligan et al., 1996). Briefly, IL-1 α , IL-2, IL-4, IL-6 and GM-CSF were assessed for the ability to support growth of LM-1, HT-2, CTLL, 7TD1 and DA-1k cell lines, respectively. Bioassay for murine IFN γ involved assessing the antiviral activity of IFN γ after stimulating L929 cells to resist infection with vesicular stomatitis virus (VSV). TNF α mediated L929 cell lysis was utilized to determine the presence and biological function of this cytokine. A biological assay of IL-10 was not carried out, however, a capture ELISA performed on media harvested from pSLRSV.IL-10 transfected COS-7 cells verified the presence of this murine cytokine. In each instance transfection samples were assessed relative to media harvested from COS-7 cells transiently transfected with a control pSLRSV.Nul plasmid.

4.2.7 Mouse injections

Inbred 7 week old, female C3H/HeN mice were injected with plasmid DNA purified by anion-exchange column chromatography and dissolved in normal saline. All injections of DNA were i.m. while injections of purified antigen (authentic or recombinant truncated gD) formulated in either VSA3 (Biostar, Saskatoon, Saskatchewan, Canada) or alum (Alhydrogel[™], Cedarlane Laboratories, Ltd., Hornby, Ontario) were subcutaneous (s.q.). Mice were physically restrained and injected using a 0.5 mL disposable Monoject® (Sherwood Medical, St. Louis, MO) syringe with a 29 gauge needle. All mice received doses of plasmid DNA split between the left and right

quadriceps muscle mass. Mice were boosted at 14 days unless otherwise stated.

Nonlethal tail bleeds were carried every 2 weeks until the experiments were terminated.

Mice were euthanized by Halothane™ (MTC Pharmaceuticals, Cambridge, Ontario) overdose.

4.2.8 Splenic and lymph node cell harvests

Mouse splenocytes were prepared by mechanically grinding spleens through a sterile nylon mesh into a sterile Petri dish containing 10-20 mL of RPMI 1640 (Sigma Chemical Co.) This material was then pipetted into sterile 15 or 50 mL tubes and allowed to sit on ice for 10 minutes. Debris that settled to the bottom was aspirated with a sterile Pasteur pipette and discarded. Tubes were centrifuged at 250 x g, in a Beckman GPR centrifuge, for 10 minutes (at 4 °C). Red blood cells were lysed by resuspension of the cell pellet in 1.0 mL/spleen of ammonium chloride lysis buffer (0.14 M NH₄Cl and 0.017 M Tris-HCl, pH 7.2) for 60 seconds. Lysis was terminated by addition of 14 mL of RPMI 1640 at room temperature. Samples were pelleted by centrifugation (250 x g) and resuspended in RPMI 1640. Splenocyte numbers were assessed by staining with Trypan blue (GIBCO/BRL) and counting on a haemocytometer. Splenocytes were centrifuged (250 x g) and resuspended at a final concentration of 1×10^7 cells/mL in RPMI 1640 supplemented with 10% (FBS) (Sigma Chemical Co.), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma Chemical Co.), 2 mM L-glutamine (GIBCO/BRL) 1mM sodium pyruvate (GIBCO/BRL), 10 mM HEPES (GIBCO/BRL) and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.)(complete RPMI 1640). A modification of a

method for harvesting Peyer's patch lymphocytes was utilized to prepare single cell suspensions from lymph nodes (Spalding, 1983). Briefly, iliac lymph nodes were collected into 15 mL tubes containing sterile chilled Phosphate Buffered Saline without calcium or magnesium chloride (PBSA)(GIBCO/BRL). Pooled nodes were placed into a sterile Petri dish and excess PBSA discarded. Nodes were diced with a sterile scalpel in a small volume of CMF solution (0.1 x Hanks Balanced Salt Solution, Ca^{2+} Mg^{2+} free; 10 mM HEPES, pH 7.2; 25 mM NaHCO_3 , pH 7.2; and 2% FBS) and then mixed with 10 mL digestion buffer (1 x Hanks Balanced Salt Solution, 10% FBS, 15 mM HEPES, pH 7.2) containing 150 Units/mL of Collagenase (CLS 1, Worthington Biochemical Corporation), and 0.015 mg/mL DNase I (Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada). Diced nodes in digestion buffer were transferred to silanized 25 -50 mL Erlenmeyer flasks, containing a sterile Teflon coated stir bar, and incubated at 37 °C, with stirring, for 90 minutes. Samples were harvested into sterile 15 mL plastic tubes and allowed to sit for 10 minutes. Large pieces of undigested material were returned to the Erlenmeyer flask and incubated an additional 30-60 minutes with 5.0 mL of digestion buffer. This material was combined with the initial harvest and cells were centrifuged (250 x g) to pellet. Digestion buffer was decanted and the cells were resuspended in PBSA (GIBCO-BRL). Cells were counted using a haemocytometer and centrifuged (250 x g) one final time. Cells were resuspended to a final concentration of 1×10^7 cells/mL.

A modified protocol for splenocytes was also used that included an additional 60 minute digestion of pooled splenocytes with collagenase (150 Units/mL of digestion buffer; Sigma Chemical Co.) and DNase I (0.015 mg/mL of digestion buffer; Pharmacia

Biotech Inc.). These digestions were only carried out in experiments that included assessment of antigen-specific responses from cells prepared from lymph nodes. These digestions were carried out prior to cell counts and resuspension.

4.2.9 Total IgG and IgG isotype ELISA

Immunolon 2 microtitre plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with purified recombinant truncated gD (Biostar Inc.) at a concentration of 0.050 µg/well. Coating antigen dilutions were done in ELISA Coating buffer (.012 M Na₂CO₃, .038 M NaHCO₃, pH 9.6) and coating was allowed to proceed overnight at 4 °C. Plates were washed 5 times in phosphate buffered saline (.137 M NaCl, .003 M KCl, .008 M Na₂HPO₄, .001 M NaH₂PO₄) with 0.05% Tween-20 (PBST) prior to addition of fourfold dilutions of mouse sera prepared in PBST with 0.5% gelatin (PBST-g), (Bio-Rad Laboratories Ltd., Mississauga, Ontario). After a 2 hour incubation, plates were washed in PBST and an affinity purified biotinylated goat antimouse Ig (Zymed Laboratories Inc., San Francisco, CA) diluted to 1/5,000 in PBST-g was added to each plate. After an incubation of 60 minutes, plates were washed extensively and Streptavidin-alkaline phosphatase (Gibco, Life Technologies, Burlington, Ontario) diluted to 1/2000, in PBST-g, was added to each plate. Following a 60 minute incubation, plates were washed six times in PBST. Prior to addition of substrate the plates were washed two additional times in PBS. Development of plates involved the addition of .01 M p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in substrate buffer (0.104 M Diethanolamine-Sigma Chemical Co., 0.5 mM MgCL₂). Absorbances were read on a Model 3550

Microplate Reader (Bio-Rad Laboratories Ltd.) at 30 minutes and again at 60 minutes at 405 nm with a reference wavelength of 490 nm.. Antibody isotyping ELISAs were carried out in a similar fashion except that biotinylated goat antimurine IgG₁ and IgG_{2a} (Caltag Laboratories, San Francisco, CA) were used at a dilution of 1/8,000 except for IgM (1/2,000). Incubation times with these antibodies was 60 minutes. Addition of Streptavidin-alkaline phosphatase and development of plates was as described for total IgG ELISA.

4.2.10 ELISPOTs

A cytokine specific ELISPOT assay was used as described previously (Czerkinsky et al., 1988; Baca-Estrada et al., 1996). Briefly, single cell suspensions isolated from spleens, lymph nodes or bone marrow were stimulated *in-vitro* by incubating in complete RPMI 1640 at 37 °C and 5% CO₂ for 20 hours in the presence of recombinant authentic gD (0.4 µg/mL). After antigen stimulation, cells were washed twice in complete media and diluted to a concentration of 1 x 10⁷ cells/mL of complete RPMI-1640. Nitrocellulose plates (FILTApate™, 0.45 µm; Polyfiltronics, Inc., Rockland, MA, USA) were prepared by coating overnight at 4 °C with 2.5 µg/mL of purified antimurine interleukin (IL)-4 (Cat. No. 11B11) or 5 µg/mL of purified antimurine interferon (IFN)-γ (Cat No. R4-6A2) (Pharmingen, San Diego, CA, USA) diluted in carbonate/bicarbonate buffer (45.3 mM NaHCO₃, 18.2 mM Na₂CO₃, pH 9.6). After coating, unbound antibody was washed from the wells thrice with sterile PBST followed by 3 washes with sterile PBS. Wells were blocked for 2 hours at 37 °C with complete RPMI 1640. Blocking

media was decanted and 100 μ L of antigen stimulated cell suspensions were added to triplicate wells. After a 20 hour incubation, at 37 °C and 5% CO₂, plates were washed 2 times in tapwater and 5 times in PBST to remove cells and nonspecifically bound cytokine. One hundred μ L of biotinylated antimurine IL-4 (Cat. No. BVD6-24G2) and IFN- γ (Cat. No. XMG1.2) (Pharmingen Canada Inc.) monoclonal antibodies (3 μ g/mL of each in 1% FBS/PBS) were added to appropriate wells and incubated for 4-6 hours at ambient temperature on an oscillating mixer. Plates were washed 6 times in PBST and 100 μ L of a 1/1000 dilution of Streptavidin alkaline phosphatase (Bio/Can Scientific, Mississauga, Ontario, Canada) in 1% FBS/PBS was added to each well. After a 2 hour incubation, at ambient temperature, the plates were washed as before twice in PBS. Plates were developed by the addition of 50 μ L of the substrates, 5-bromo-4-3-indolyl phosphate (BCIP), and nitroblue tetrazolium (NBT), (Moss Inc., Pasadena, MD). Plates were allowed to develop at ambient temperature for 10-30 minutes, after which reactions were stopped by extensive washing in distilled water. After air drying, spots were counted under a dissecting microscope. B-cell ELISPOT involved the use of nitrocellulose plates (Millipore Milliscreen-HA, Millipore, Bedford, MA) that were coated overnight at 4 °C with purified recombinant truncated gD at 5.0 μ g/mL in carbonate/bicarbonate buffer (pH 9.6). Coated plates were washed three times in sterile PBST followed by 3 washes in sterile PBS. Nonspecific binding sites were then blocked with complete RPMI 1640 at 37 °C for 1-2 hours. Fresh, unstimulated cells from spleen or lymph nodes, prepared as described above, were added in a volume of 100 μ L (1 x

10^7 cells/mL) to each well. Uncoated but blocked control wells, for nonspecific binding of IgG, also received cells from each test sample in triplicate. Plates were incubated at 37 °C with 5% CO₂ for 10-12 hours. After the incubation period, all cells were washed out of the wells by 2 initial washes with tapwater followed by 6 washes with PBST. Biotinylated antimurine IgG₁ or IgG_{2a} antibodies (1/8,000 dilution) were added in a 100 µL volume of PBST (with 1% FBS) and incubated with mixing for 3-4 hours at ambient temperature. After washing in PBST six times, streptavidin-alkaline phosphatase (1/1,000 dilution in PBS-T with 1% FBS) was added to each well (100 µL). After a 60 minute incubation, plates were washed six times in PBST and 2 times in PBS. Plates were developed as described for the cytokine ELISPOT.

4.2.11 Statistical analysis

Data was entered into a database in the statistical analysis program Instat GraphPad™. Differences in antibody titres among vaccine groups were investigated using the nonparametric Mann-Whitney *U* test.

4.3 Results

4.3.1 Immunization with different doses of pSLRSV.SgD.

In order to identify a suboptimal dose that would subsequently be utilized in cytokine modulation experiments a DNA dose titration experiment was conducted. The impact of single dosing versus boosting with pSLRSV.SgD on serum IgG titres,

seroconversion efficacy and splenic cytokine profiles was also determined. Female C3H/HeN mice were immunized i.m. either once (single dose group) or twice (boosted group) with either 2, 10 or 50 µg of pSLRSV.SgD. Saline (SAL) and conventional vaccine (VSA3 + tgD) groups received boosts 14 days following the initial immunization. Mice were euthanised at 2, 4 and 6 weeks and spleens harvested, pooled and assessed for IL-4 or IFN γ secretion as measured by ELISPOT. We had previously demonstrated that most mice immunized and boosted with 100 µg of pSLRSV.SgD seroconverted at 2 weeks with peak serum IgG titers exceeding 40,000 in some instances (section 3.3.2). We also demonstrated previously that mice immunized with pSLRSV.SgD displayed measurable serum titers 5 1/2 months following boost and that predominant serum isotype was IgG₁. Despite the predominance of a serum isotype that correlates to a Th2 type of immune response the 6 month splenic cytokine profile, following restimulation *in vitro*, was almost exclusively IFN γ. Upon further examination we were able to demonstrate that the predominant serum isotype in mice immunized two times correlates more closely to the cytokine profile in the iliac lymph node, which drains the deep tissues of the quadriceps muscle mass.

This dose titration experiment (Tables 4.2 and 4.3) clearly shows that 2 to 4 week serum antibody levels in mice immunized with a single dose of pSLRSV.SgD were greatly influenced by the dose of DNA. One hundred percent of mice immunized with a single 50 µg dose of pSLRSV.SgD seroconverted with mean titers at 2 weeks significantly ($p \leq 0.5$) higher than in mice receiving lower doses. A single 10 µg dose of

Table 4.2. Dose Titration of a single dose of pSLRSV.SgD.

Vaccine Groups ^a	Dose ^b (μ g)	Serum ELISA Titers ^c (no. seropositive mice/no. immunized)			Splenic Cytokine ^d Profile (6 week)	
		2 weeks	4 weeks	6 weeks	IFN γ	IL-4
DNA	2	67 \pm 11 (0/15)	170 \pm 71 (1/10)	290 \pm 142 (2/5)	24 \pm 3	10 \pm 4
DNA	10	1187 \pm 436 (5/15)	5128 \pm 2944 (4/9)	8867 \pm 8368 (2/3)	30 \pm 2	14 \pm 4
DNA	50	9707 \pm 2015 (15/15)	8640 \pm 2339 (10/10)	9600 \pm 4411 (5/5)	49 \pm 6	14 \pm 2
VSA3 + tgD	.05	110 \pm 16 (0/15)	n.d.	n.d.	n.d.	n.d.
VSA3 + tgD	.25	147 \pm 25 (1/15)	n.d.	n.d.	n.d.	n.d.
VSA3 + tgD	1.25	310 \pm 207 (1/15)	n.d.	n.d.	n.d.	n.d.
SAL	-	46 \pm 13 (0/15)	n.d.	n.d.	n.d.	n.d.

^a DNA is plasmid pSLRSV.SgD which encodes a secreted form of BHV-1 gD

^b DNA and VSA3/tgD were administered i.m. or subcutaneously, respectively

^c ELISA titers are represented as the mean value of serum titers from seropositive mice (≤ 200 is seronegative) with standard error of the mean depicted. Values in parentheses beneath ELISA titers represents the number of seropositive mice within the total number of mice immunized at day 0. Five mice from each of the DNA groups were euthanized at 2, 4 and 6 weeks for splenic cytokine determination. Values in parentheses at 4 and 6 weeks represent the number of seropositive mice in the remaining mice within the group.

^d Values represent the mean number of cytokine secreting cells determined from triplicate wells. Standard deviations are indicated

n.d. - not done

Table 4.3. Dose Titration of pSLRSV.SgD in Mice after Boosting

Vaccine ^a Groups	Dose ^b (μ g)	Serum ELISA Titers ^c (no. seropositive mice/no. immunized)			Splenic Cytokine ^d Profile (6 week)	
		2 weeks	4 weeks	6 weeks	IFN γ	IL-4
DNA	2	117 \pm 22 (1/15)	200 \pm 100 (1/15)	540 \pm 332 (2/10)	n.d.	n.d.
DNA	10	3053 \pm 335 (10/15)	5813 \pm 1746 (15/15)	7120 \pm 2480 (10/10)	37 \pm 4	14 \pm 2
DNA	50	4147 \pm 1643 (13/15)	17500 \pm 3922 (14/15)	9360 \pm 2225 (10/10)	24 \pm 5	11 \pm 3
VSA3 + tgD	.05	110 \pm 16 (0/15)	55 \pm 5 (0/10)	200 \pm 55 (1/5)	n.d.	n.d.
VSA3 + tgD	.25	146 \pm 25 (1/15)	130 \pm 33 (1/10)	140 \pm 25 (0/5)	n.d.	n.d.
VSA3 + tgD	1.25	310 \pm 207 (1/15)	1150 \pm 613 (5/10)	760 \pm 610 (1/5)	13 \pm 1	21 \pm 8
SAL	-	70 \pm 7 (0/15)	120 \pm 19 (0/10)	180 \pm 20 (0/10)	2 \pm 2	4 \pm 2

^a DNA is plasmid pSLRSV.SgD which encodes a secreted form of BHV-1 gD

^b DNA and VSA3/tgD were administered i.m. or subcutaneously, respectively. All mice were boosted 14 days after the initial immunization.

^c ELISA titers are represented as the mean value of serum titers from seropositive mice (\leq 200 is seronegative) with standard error of the mean depicted. Values in parentheses beneath ELISA titers represents the number of seropositive mice within the total number of mice immunized. Each vaccination started with 15 mice per group. Five mice from VSA3/tgD groups were euthanized at 2, 4 and 6 weeks for splenic cytokine evaluation. Five mice from the DNA groups were euthanized at 4 and 6 weeks for splenic cytokine determination.

^d Values represent the mean number of cytokine secreting cells determined from triplicate wells. Standard deviations are indicated

n.d. - not done

pSLRSV.SgD was suboptimal as seen by poor seroconversion efficiency in immunized mice. Mean serum titers, at 2 weeks, of mice immunized with this dose of DNA were significantly ($p \leq 0.05$) lower than mice immunized with 50 μg of DNA. However, mean titers in seropositive mice were not significantly ($p \leq 0.05$) different at 4 or 6 weeks between these two groups. Only two mice of 15, receiving a single dose of 2 μg of pSLRSV.SgD, showed evidence of seroconversion at 6 weeks post immunization. Despite the absence of serum titers in mice receiving 2 μg of vaccine there was clear evidence of T-cell priming with low, but significant, levels of IFN γ and IL-4 production in 6 week splenocytes. The predominant T-cell response in all DNA vaccination groups appeared to be of the Th1-type phenotype as indicated by a 2-3 fold higher level of IFN γ secretion as compared to IL-4. Table 4.3 summarizes the kinetics of the antibody response and splenic cytokine profiles of C3H/HeN mice boosted 14 days after the initial immunization. Mice boosted with 2 μg of pSLRSV.SgD showed a pattern similar to mice immunized one time only, in that seroconversion efficacy was very low, although titers in the few boosted seropositive mice tended to be slightly higher than mice receiving a single low dose of pSLRSV.SgD. Mice boosted with 10 μg of pSLRSV.SgD show little difference in mean serum antigen-specific antibody titres at 4 and 6 weeks as compared to their single dosed seropositive counterparts. Surprisingly the 2 week titers of mice boosted at day 14 was significantly ($p \leq 0.05$) higher than the mice in the single dose group. This discrepancy between groups of mice immunized one time with the same dose of DNA prepared from the same batch of plasmid was somewhat surprising and we can only attribute the differences in early mean serum titers in these 10 μg dose groups on

variability of the injection technique. Despite the 2 week titer differences in these two groups of mice the primary difference appears to be the enhanced efficacy of seroconversion observed in the boosted group (100%) at 4 weeks as compared to the single dose group (40%). Finally, mice boosted with 50 ug of pSLRSV.SgD displayed mean serum titers that were consistently higher than those observed in mice immunized with lower doses of DNA. However, only 4 week titers in mice immunized and boosted with 50 ug of pSLRSV.SgD are significantly higher ($p \leq 0.05$) than mice boosted with 10 ug of the vaccine. There appears to be no early immunological benefit observed in mice immunized once or twice at the optimal dose of pSLRSV.SgD (50 ug). We included a conventional vaccine comprised of affinity column purified recombinant truncated gD (tgD) formulated with the adjuvant VSA3. This conventional vaccine formulation has been shown to be highly efficacious in cattle and at least two other strains of mice (C57BL/6 and Balb/c) and typically elicits a predominance of IL-4 in antigen restimulated splenocytes (van Drunen Littel-van den Hurk et al., 1984; Baca-Estrada et al., 1996). We included this conventional vaccine as a Th2-type positive control group for DNA-immunized mice which typically show a Th1-type cytokine profile in antigen restimulated splenocytes (section 3.3.4). Despite this we have been plagued by highly inconsistent immune response to this vaccine formulation in C3H/HeN (H-2^k) mice. Doses between 100 and 1000 ng consistently yielded excellent serum titers, following a boost, in C57BL/6 (Baca-Estrada et al., 1996). Despite this, Table 4.3 shows evidence that C3H/HeN mice are poor, or inconsistent, responders to a substantial dose range of recombinant tgD formulated in potent adjuvant. However, we assessed splenic cytokine

profiles in the few seropositive mice immunized and boosted with the highest dose of VSA3/tgD. Although there were some IFN γ secreting splenocytes there were 2 fold higher numbers of IL-4 secreting cells upon *in vitro* restimulation of splenocytes harvested from these high dose VSA3/tgD mice. Although the numbers are not nearly as high as observed in other experiments the tendency for this vaccine formulation to drive a Th2 type of immune response is consistent.

4.3.2 Co-administration of pSLRSV.SgD and plasmids encoding cytokines.

The objective of this experiment was to determine which of eight different murine cytokines, encoded within expression plasmids and co-administered with a suboptimal (10 μ g) of pSLRSV.SgD (Fig. 4.2), would enhance or modulate the immune response. Since excessive doses of some of the proinflammatory cytokines (IL-1, IL-6, TNF α) might invoke a clinical response and hinder interpretation of the results a dose of 10 μ g of pSLRSV.SgD was used. This dose of vaccine was capable of eliciting reasonable titers in 40-60% of immunized mice. A dose of 10 μ g of plasmids encoding cytokines was chosen for this preliminary experiment based on the observation that ~50% of mice were capable of responding to an identical dose of pSLRSV.SgD. Figs 4.3, A, B, and C summarize the mean total IgG anti-gD serum titers for immunized mice at 2, 4 and 6 weeks post-immunization. At 2 weeks, mice immunized once with pSLRSV.GM-CSF, pSLRSV.IL-4 and pSLRSV.TNF α all show mean serum titers that were significantly ($p \leq 0.05$) higher than control mice immunized with 10 μ g of pSLRSV.SgD co-delivered with pSLRSV.Nul-B. Table 4.4 shows that at 2 weeks 40% of mice seroconverted when

Fig. 4.2 Diagrammatic characterization of the plasmid co-administration protocol. Vector pSLRSV.SgD, which encodes a secreted version of bovine herpesvirus (BHV)-1 glycoprotein D (gD), was mixed with each of eight different murine cytokine cDNAs in the context of the pSLRSV expression cassette. All vaccine mixtures were dissolved in normal saline in a final volume of 100 μ L. The dose was then split and 50 μ L injected into both quadriceps muscle masses.

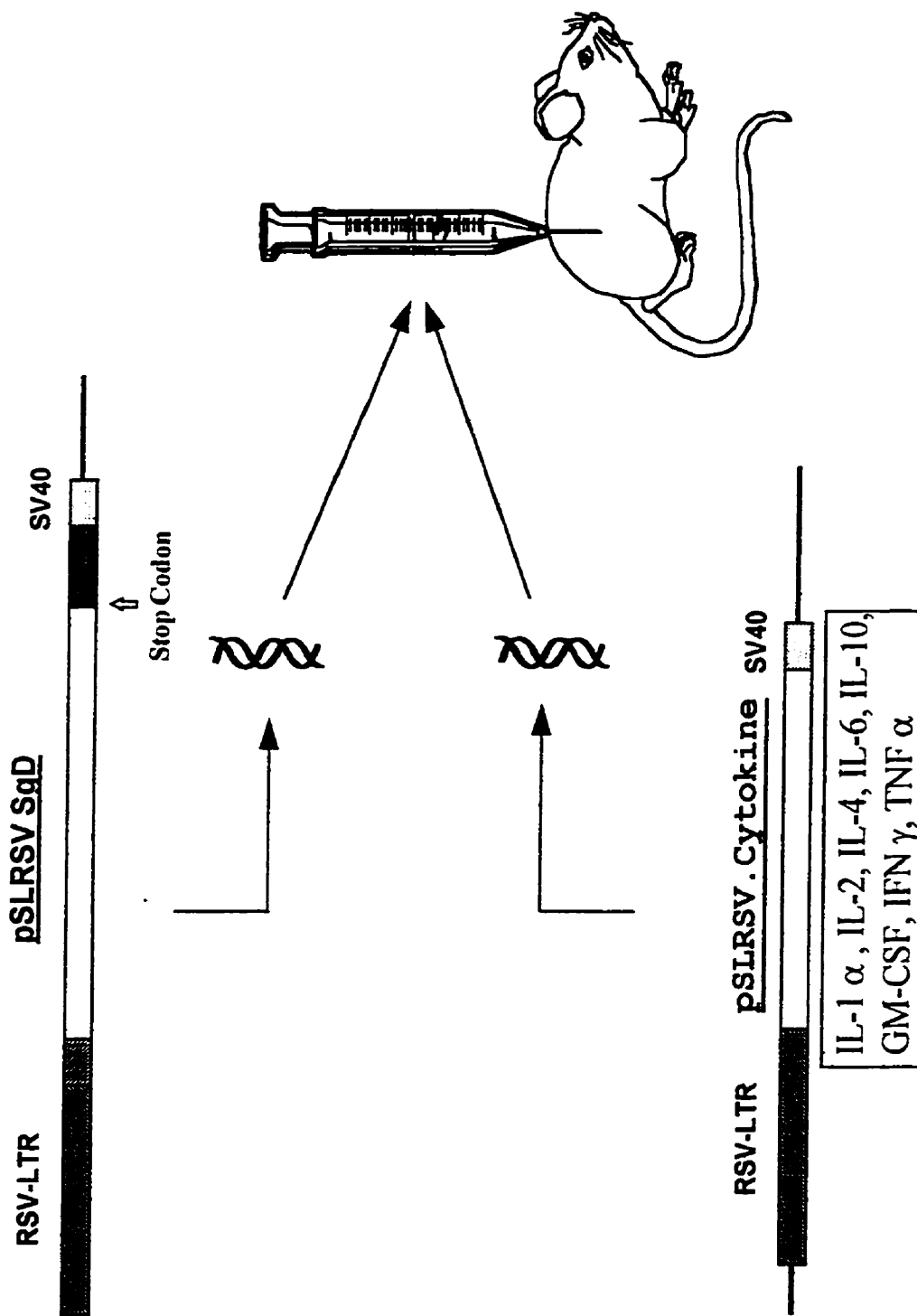


Fig. 4.3 Effect of plasmids encoding cytokines on immune responses to a DNA vaccine. Mean ELISA titers for serum IgG in mice immunized once with pSLRSV.SgD and an expression cassette encoding one of eight different murine cytokines. Groups of female C3H/HeN mice (9 or 10/group) were immunized with a suboptimal dose (10 µg/mouse) of pSLRSV.SgD co-administered with 10 µg of expression vectors encoding: IL-1 α , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN γ or TNF α . Control groups included: mice immunized with a mixture of pSLRSV.SgD and pSLRSV.Nul, as well as, a conventional vaccine formulation comprised of VSA3 formulated with a recombinant secreted version of BHV-1 gD (500 ng/mouse). Data presented depicts total IgG in sera harvested at 2 (**A**), 4 (**B**) and 6 (**C**) weeks.

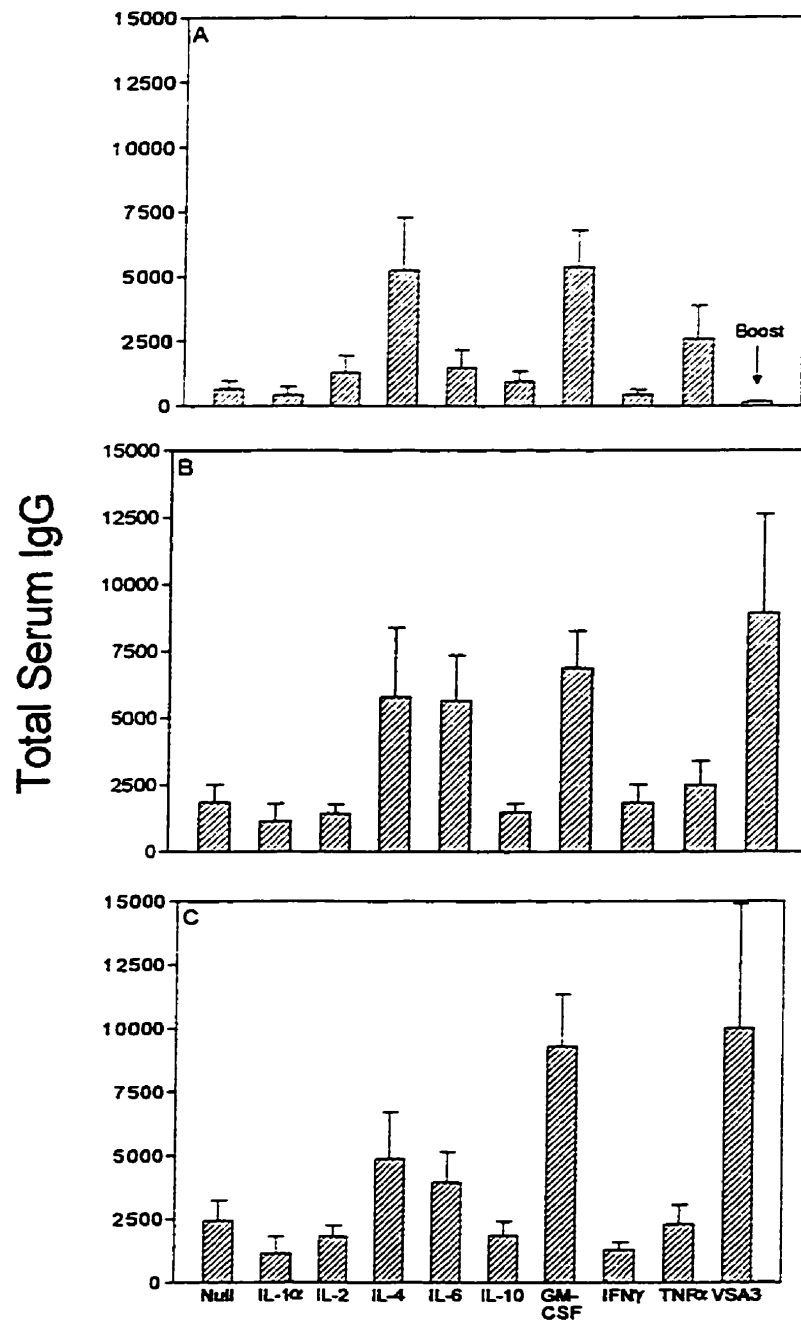


Table 4.4 Mean serum titers following co-administration of plasmids encoding cytokines

Vaccine ^a Groups	Serum ELISA Titers ^b (no. of seropositive mice/total no. of mice immunized)					
	2 weeks		4 weeks		6 weeks	
pSLRSV.Nul	650 ± 323	(4/10)	1860 ± 669	(6/10)	2440 ± 786	(6/10)
pSLRSV.IL-1 α	420 ± 310	(2/10)	1150 ± 660	(4/10)	1145 ± 661	(4/10)
pSLRSV.IL-2	1295 ± 648	(5/10)	1420 ± 355	(8/10)	1815 ± 419	(8/10)
pSLRSV.IL-4	5240 ± 2058	(6/10)	5790 ± 2597	(5/10)	4860 ± 1846	(5/10)
pSLRSV.IL-6	1500 ± 679	(4/10)	5645 ± 1725	(7/10)	3935 ± 1213	(8/10)
pSLRSV.IL-10	955 ± 387	(6/10)	1485 ± 335	(9/10)	1845 ± 570	(5/10)
pSLRSV.GM-CSF	5370 ± 1437	(9/10)	6880 ± 1392	(10/10)	9280 ± 2049	(10/10)
pSLRSV.IFN γ	444 ± 177	(4/10)	1828 ± 700	(6/10)	1278 ± 314	(7/10)
pSLRSV.TNF α	2585 ± 1300	(6/10)	2515 ± 899	(7/10)	2285 ± 773	(9/10)
VSA3/tgD	140 ± 45	(2/10)	8925 ± 3688	(7/10)	10010 ± 4874	(9/10)

^a Mice were immunized as described in Fig. 4.3. Plasmids encoding cytokines (10 μ g) were mixed with 10 μ g of pSLRSV.SgD and injected i.m. in the quadriceps muscles (doses were split between left and right quadriceps). Conventional vaccine (VSA3/tgD) was administered subcutaneously as described in Methods (section 4.2.7) and boosted at 14 days. Mice receiving DNA-based vaccines were immunized once only.

^b ELISA titers values were determined by taking the mean of all mice in each group. Deviations are indicated as standard error of the mean. Values in parentheses represent the number of seropositive mice within the total number of mice immunized.

vaccinated with pSLRSV.SgD + pSLRSV.Nul-B. Seroconversion efficacy ranged from 20% (pSLRSV.IL-1 α and VSA3/tgD) to 60% (pSLRSV.IL-4, pSLRSV.IL-10 and pSLRSV.TNF α) depending on the cytokine construct. These data suggest that these cytokines (pSLRSV.IL-1 α , pSLRSV.IL-4, pSLRSV.IL-10 and pSLRSV.TNF α) had little

impact on seroconversion efficiency when co-administered with a suboptimal dose of pSLRSV.SgD. Conversely, mice immunized with pSLRSV.SgD + pSLRSV.GM-CSF showed both the highest mean serum anti-gD IgG titers and seroconversion levels of 90 % and 100%, at 2 and 4 weeks, respectively. At 4 weeks post-immunization, mean titers in all but one vaccine group (pSLRSV.TNF α + pSLRSV.SgD) had increased. Mice boosted, at day 14, with VSA3/tgD displayed the highest mean serum IgG titers, however, the mean titers in this group are not statistically ($p < 0.05$) different from mean titers observed in mice immunized once with suboptimal doses of pSLRSV.SgD + pSLRSV.GM-CSF, pSLRSV.IL-4 or pSLRSV.IL-6 (Table 4.4). Seroconversion efficacy of the VSA3/tgD group (70 %) at 4 weeks resembled that seen in mice immunized with pSLRSV.SgD + pSLRSV.IL-4 or pSLRSV.IL-6 (50 and 60 %, respectively). Notably, 100% of mice immunized once, with a suboptimal dose of pSLRSV.SgD + pSLRSV.GM-CSF seroconverted by 4 weeks post-immunization.

Comparisons of DNA-based vaccine + plasmids encoding cytokines with VSA3/tgD is of some importance for several reasons. First, a suboptimal single dose of a DNA-based vaccine co-administered with plasmids encoding murine GM-CSF elicited serum antibody levels that essentially equalled an optimized dose (for C57BL/6 and Balb/c) of recombinant tgD formulated with a potent adjuvant. Second, seroconversion efficacy responses to VSA3/tgD varied significantly in C3H/HeN mice while co-administration of pSLRSV.GM-CSF with 10 μ g of pSLRSV.SgD resulted in 100% seroconversion at 4 weeks. There was a general increase in 4 week serum IgG titers (slight increase in pSLRSV.SgD + pSLRSV.IL-2, pSLRSV.IL-4, or pSLRSV.GM-CSF) 1.5 - 2 fold

increases in pSLRSV.SgD + pSLRSV. IL-1 α and pSLRSV.IL-10 groups, and a 2.5 - 4 fold increases in pSLRSV.SgD + pSLRSV.Nul, pSLRSV.IL-6 or pSLRSV.IFN γ groups. All vaccine groups, except SgD/IL-4 (10% decrease), showed increases in the number of seropositive mice ranging from 10% (SgD/GM-CSF; SgD/TNF α) to 50% (VSA3/tgD). Fig. 4.3 C and Table 4.4 summarize mean serum titer IgG titers at 6 weeks post-immunization (4 weeks postboost for the VSA3/tgD vaccine group). Due to a slight decrease in the mean serum titers for pSLRSV.SgD + pSLRSV.IL-4 and pSLRSV.IL-6 groups only mice immunized with pSLRSV.SgD + pSLRSV.GM-CSF showed titers that were statistically higher ($p \leq 0.05$) than all other groups immunized with DNA-based vaccine formulations. At 6 weeks post-immunization the mean serum IgG titers for mice immunized, and boosted, with VSA3/tgD were not significantly different from mean titers observed in the pSLRSV.SgD + pSLRSV.GM-CSF group.

4.3.3 Isotype profiles following co-administration of pSLRSV.SgD with plasmids encoding cytokines.

To further characterize the immune response to plasmids encoding cytokines co-administered with a suboptimal dose of pSLRSV.SgD, we assessed the serum levels of IgG₁ and IgG_{2a} in mice. Fig. 4.4 shows mean IgG₁ and IgG_{2a} isotype values for seropositive mice at 6 weeks post-immunization. Typically mice immunized with pSLRSV.SgD showed an IgG₁: IgG_{2a} ratio 2.0 - 2.5: 1 (Fig. 4.5). Isotype data for the pSLRSV.SgD + pSLRSV.Nul group were consistent with previous observations (section 3.3.3). Fig. 4.4 and Table 4.5 summarize the mean isotype values determined from the 6

Fig. 4.4 Serum IgG isotype profile after immunization with plasmids encoding antigen and cytokines. Mean levels of anti-gD IgG₁ and IgG_{2a} antibodies in seropositive C3H/HeN mice immunized intramuscularly once with pSLRSV.SgD co-administered with an expression vector encoding one of eight different murine cytokines. Cytokines co-administered with pSLRSV.SgD are indicated along the x-axis. The conventional (VSA3 + recombinant, secreted form of gD) vaccine group and a control group immunized with pSLRSV.Nul (Null) + pSLRSV.SgD are also depicted. In all cases, with the exception of the VSA3 group, mice received a single, suboptimal dose of plasmids encoding SgD and one of the eight murine cytokines. Immunization doses are as indicated in Fig. 4.3. Isotype levels are depicted as optical densities at a 1/100 dilution of 6 week, post immunization, sera following a 30 minute development in an Immulon 2U ELISA plate with the substrate p-nitrophenyl phosphate.

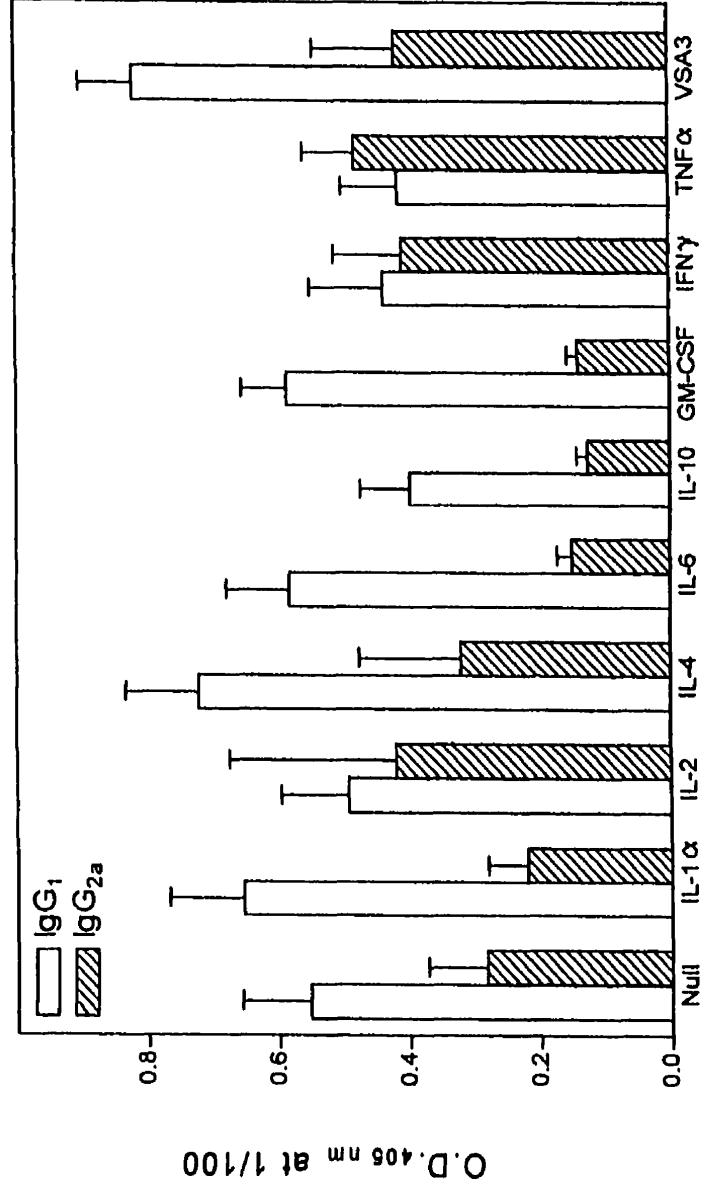


Table 4.5 Serum isotype profile after co-administration of plasmids encoding cytokines and a secreted form of BHV-1 gD.

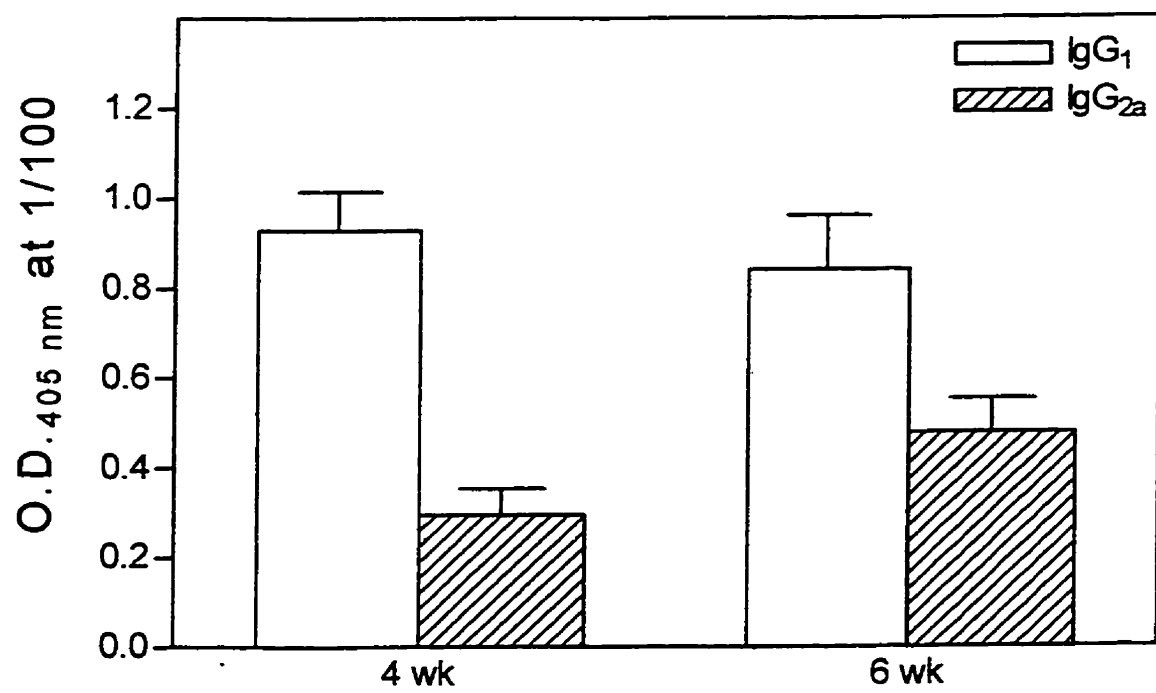
Vaccine ^a Groups	# Mice With ^b Predominance of Serum IgG ₁	Mean Isotype O.D. (1/100) ^c		Ratio of IgG ₁ :IgG _{2a}
		IgG ₁	IgG _{2a}	
SgD Only	6/7	.6511 ± .1476	.4491 ± .1300	1.45
SgD + Null	5/6	.5523 ± .1045	.2833 ± .0899	1.95
SgD + IL-1 α	4/4	.6540 ± .1134	.2205 ± .0602	2.96
SgD + IL-2	3/5	.4934 ± .1032	.4202 ± .2550	1.17
SgD + IL-4	4/5	.7220 ± .1112	.3206 ± .1559	2.25
SgD + IL-6	8/8	.5825 ± .0964	.1496 ± .0229	3.89
SgD + IL-10	8/9	.3983 ± .0757	.1260 ± .0168	3.16
SgD + GM-CSF	10/10	.5865 ± .0698	.1404 ± .0162	4.19
SgD + IFN γ	3/7	.4381 ± .1115	.4084 ± .1043	1.07
SgD + TNF α	3/5	.4154 ± .0861	.4810 ± .0779	0.86
VSA3/tgD	8/9	.8196 ± .0821	.4187 ± .1251	1.96

^a SgD= pSLRSV.SgD, all cytokines are as depicted in Table 4.4 (ie. plasmid encoded). Null refers to the Null plasmid pSLRSV.Null. The conventional vaccine VSA3/tgD has been described in Methods (section 4.2.7) and the dose is indicated in the legend to Fig. 4.3. Doses and co-administration of plasmids has been described in Methods, the legend to Fig. 4.3 and in the footnotes to Table 4.4.

^b Levels of immunoglobulin isotypes were determined by ELISA as described in Methods (section 4.2.9). Data refers to the number of mice showing a predominance, not exclusivity, of serum IgG₁ for all seropositive mice at 6 weeks. Predominance was determined by the magnitude of O.D._{405 nm} values for IgG₁ and IgG_{2a} at a 1/100 dilution.

^c Data is represented as the mean of O.D._{405 nm} values for IgG₁ or IgG_{2a} in all seropositive mice.

Fig. 4.5 Serum IgG isotype profile after immunization with plasmids encoding a secreted form of BHV-1 gD. Relative amounts of IgG₁ and IgG_{2a} in mice immunized with 50 µg of pSLRSV.SgD and boosted at 2 weeks. Values are presented as optical densities (405 nm) following development of ELISA plates in the presence of the substrate p-nitrophenyl phosphate. All values were determined at a serum dilution of 1/100 and for mice 2 weeks or 4 weeks post boost. Mean values were calculated from optical densities determined for 9 (4 weeks) or 10 (6 weeks) individual mice.



week sera of seropositive mice. Co-administration of Th2-associated cytokines, including IL-10, IL-6 and IL-4 appeared to enhance the polarization of serum IgG isotypes towards IgG₁. Surprisingly co-administration of plasmids encoding GM-CSF resulted in the most extreme polarization of serum isotypes towards IgG₁, largely by enhancing the serum level of IgG₁ without a concurrent increase in IgG_{2a}. One hundred percent of mice receiving the pSLRSV.SgD + pSLRSV.GM-CSF and pSLRSV.IL-6 vaccines were predominantly IgG₁, while mice immunized with pSLRSV.SgD + pSLRSV/IL-4 and pSLRSV.IL-10 showed 89% and 80% predominance of IgG₁, respectively. The one mouse in the pSLRSV.SgD + pSLRSV.IL-4 group which was predominantly IgG_{2a} displayed a ratio of IgG₁: IgG_{2a} of 0.464 (data not shown). C3H/HeN mice immunized with the conventional vaccine VSA3/tgD displayed a predominance of IgG₁, in serum of most mice, 4 weeks post boost. At least two high titer mice, in the conventional vaccine group showed substantial amounts of serum IgG_{2a} (IgG₁:IgG_{2a} ratios of 1.34 and 0.88)(data not shown). Mice immunized with pSLRSV.SgD in conjunction with plasmids encoding IL-2, IFN γ or TNF α showed a clear trend towards an increase in serum IgG_{2a}. In all three cases the prevalence of IgG_{2a} is inconsistent between individual mice within these three groups. Table 4.5 shows mice immunized with pSLRSV.SgD + pSLRSV.IL-2 or pSLRSV.TNF α showed a clear trend towards a predominance of serum IgG_{2a}, with mean serum IgG₁:IgG_{2a} ratios of 1.17 and 0.86 respectively. Sixty-eight percent (4 of 7 seropositive animals) of mice immunized with pSLRSV.SgD + pSLRSV.IFN γ displayed a predominance of serum IgG_{2a} although the isotype ratio derived from mean isotype levels for all seropositive animals in this

vaccine group demonstrates an overall predominance of IgG₁ (Table 4.5).

4.3.4 High and Low Doses of pSLRSV.IFN γ and pSLRSV.GM-CSF co-administered with pSLRSV.SgD.

Results summarized in Figs.4.3 and 4.4, as well as, Tables 4.4 and 4.5 suggested that co-administration of a DNA-based vaccine with plasmids encoding murine GM-CSF or IFN γ warranted further investigation. The dose of plasmids encoding cytokines were not optimized in the preliminary experiments therefore the impact of two different doses of these plasmid encoding cytokines, on the ensuing immune response to a fixed dose (50 μ g) of pSLRSV.SgD, was assessed. Fig. 4.6 A, B and C summarize the mean serum IgG titers for all mice immunized with 50 μ g of pSLRSV.SgD co-administered with 10 or 50 μ g of plasmids encoding murine GM-CSF (groups designated GS- 60 and GS-100), IFN γ (groups designated IS-60 or IS-100) or a control, pSLRSV.Nul, plasmid (groups designated NS-60 or NS-100). Mice were immunized i.m. and boosted at 4 weeks.

Two weeks post immunization the seroconversion efficacy is increased regardless of the dose of pSLRSV.GM-CSF (Table 4.6 and Fig. 4.6 A). The high dose pSLRSV.GM-CSF group (GS-100) appeared, despite 100% seroconversion, to suppress the humoral response relative to the NS-100 and IS-100 groups. High doses (NS-100) of Nul plasmid in conjunction with the fixed dose of pSLRSV.SgD also appeared to suppress the magnitude of the humoral response (Figs. 4.6 A, B and C). This "suppression" may simply reflect competition for cell surface sites of plasmid uptake or may possibly be the

result of an adjuvant effect intrinsic to bacterially derived plasmid DNA that deviates the local lymphoid microenvironment such that a potent Th1 response is facilitated (Roman et al., 1997). Interestingly, a 10 µg dose of pSLRSV.IFN γ (IS-60) appeared to suppress humoral responses as compared to NS-60 and GS-60 vaccine groups (Figs. 4.6 A, B and C). This dose of IFNγ appeared to have little effect on seroconversion efficiency relative to NS-60 and GS-60 (Table 4.6). Conversely, co-administration of a high dose (50 µg) of plasmids encoding IFN γ enhanced mean, gD-specific, serum IgG levels as compared to 2 week titers in mice immunized with pSLRSV.SgD and 50 µg doses of null plasmid (NS-100) or plasmids encoding GM-CSF (GS-100).

Data obtained from animals four weeks after immunization showed that NS-60 and GS-60 mean antibody titers were essentially equal and significantly ($p \leq 0.05$) greater than mean serum titers in the IS-60 group (Figs. 4.6 B). The advantages conferred by co-administration of pSLRSV.GM-CSF observed in Fig. 4.3 A, B, and C were less apparent in this titration experiment possibly due to the use of an essentially optimized 50 µg dose of pSLRSV.SgD. Mice immunized with pSLRSV.SgD and 10.0 µg of plasmids encoding IFN γ (IS-60) exhibit diminished mean serum titers at 4 weeks (Fig. 4.6 B). This reduction in humoral response in this vaccine group is significantly ($p \leq 0.05$) lower than mean titer values in the corresponding NS-60 and GS-60 vaccine groups. Mean serum IgG titers, at 4 weeks, in the NS-100 and GS-100 groups are significantly ($p \leq 0.05$) lower than serum titers in the IS-100 group. Despite low serum titers in these two groups, seroconversion efficacy was 100% at 4 weeks (Table 4.6).

After boosting our data demonstrated that specific vaccine groups benefited from the

Fig. 4.6 Effect of co-administration of plasmids encoding antigen and GM-CSF or IFN γ . Mean serum ELISA titers for mice immunized with a fixed dose of pSLRSV.SgD and varying doses of expression vectors encoding murine GM-CSF or IFN γ . Female C3H/HeN mice were immunized i.m. with 50 μ g of pSLRSV.SgD and 10 or 50 μ g of pSLRSV.GM-CSF, pSLRSV.IFN γ , or Null vector pSLRSV.Nul. Plasmid combinations were mixed in 100 μ L normal saline and the dose was split between both quadriceps muscle masses. Mice were boosted 4 weeks after the initial vaccination. Serum from vaccinated mice was harvested 2 (A), 4 (B) and 6 (C) weeks following the initial immunization. Total IgG titers are depicted as end point dilution values using 3 standard deviations above the mean O.D. (405 nm) of prebleed sera as a cutoff. X-axis groups represent the following individual vaccine groups: Sal. (normal Saline control), Null (control expression vector), NS-60 and NS-100 (Null vector + pSLRSV.SgD), IS-60 and IS-100 (pSLRSV.IFN γ + pSLRSV.SgD) and GS- 60 and GS-100 (pSLRSV.GM-CSF + pSLRSV.SgD). Numbers "60" and "100" within the group designations indicate final amount (in μ g) of plasmids injected: in all cases the dose of pSLRSV. SgD was fixed at 50 μ g and doses of 10 or 50 μ g of the Nul, or cytokine expressing, plasmids were added. Addition of the Nul or cytokine plasmid amounts to the fixed dose of pSLRSV.SgD generated the final dose values of 60 and 100 μ g depicted as part of the x-axis designations.

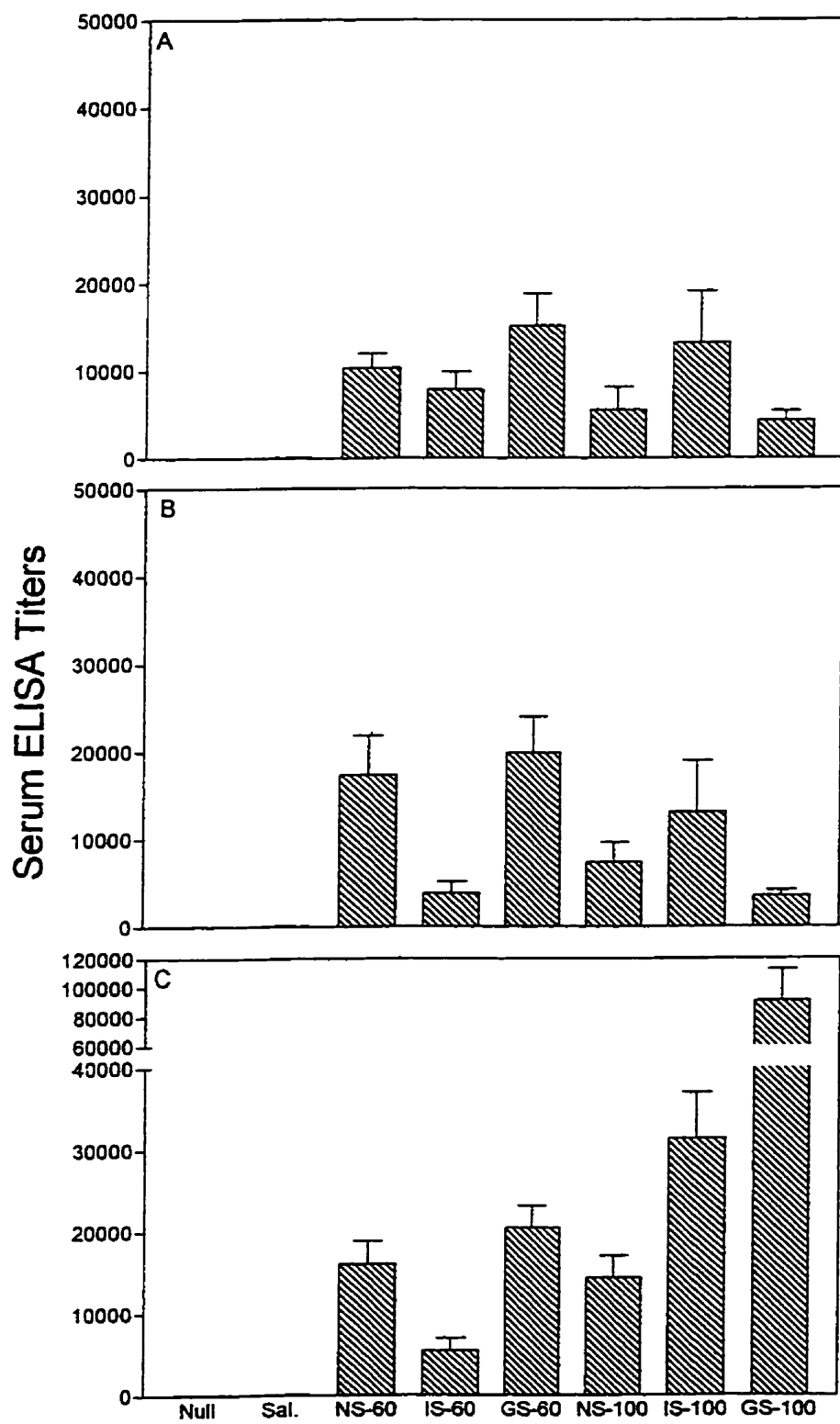


Table 4.6 Seroconversion following co-administration of pSLRSV.SgD with pSLRSV.GM-CSF or pSLRSV.IFN γ

Vaccine ^a Groups	No. of Seropositive Mice ^b		
	2 weeks	4 weeks	6 weeks
NS-60	9/10	10/10	10/10
IS-60	8/10	8/10	7/10
GS-60	10/10	10/10	10/10
NS-100	8/10	10/10	10/10
IS-100	7/9	8/9	8/9
GS-100	10/10	10/10	10/10

^a Vaccine group designations are indicated in the legend to Fig. 4.6.

^b Values represent the number of seropositive mice/total number of immunized animals

boost (Fig. 4.6). Mice immunized with pSLRSV.SgD + 50.0 μ g of pSLRSV.IFN γ or pSLRSV.GM-CSF showed mean serum IgG titers that were significantly higher ($p \leq 0.05$) than corresponding preboost (4 week) titers. Mean serum titers postboost in the NS-100 vaccine group increased as well, however, this increase was not statistically significant ($p \leq 0.05$). Interestingly, mice in the GS-100 group, that showed an apparent preboost suppression of antigen-specific humoral responses, displayed the highest post boost serum IgG titers and were statistically significant ($p \leq 0.05$). Co-administration of 10.0 μ g of pSLRSV.IFN γ appeared to be having a negative impact on the development of serum antibody titers, despite boosting, at 6 weeks (Fig. 4.6 C). Finally, vaccine groups NS-60 and GS-60 showed no apparent enhancement in mean serum titers as compared to preboost, 4 week, titers. Table 4.4 shows the number of responders at 4

weeks to be 100%, in all experimental groups except the IS-60 and IS-100 vaccine groups. Table 4.4 also shows that one mouse in the IS-60 vaccine group appeared to stop responding at 6 weeks. These transient responders showed titers just above cutoff level at 4 weeks (1/400 dilution) and may have been weak responders or simply scored as responders at 4 weeks as a result of sample variation during the ELISA plate preparation and reading. In any event these mice were not influenced in a positive manner, as determined by the absence of gD specific serum titers at 2 weeks post boost.

4.3.5 Both GM-CSF and IFN γ can increase the levels of serum IgG_{2a}.

Co-administration of 10 μ g of plasmids encoding IFN γ and GM-CSF with a suboptimal dose (10 μ g) dose of pSLRSV.SgD increased serum levels of IgG₁ and IgG_{2a}, respectively (Table 4.5). In an effort to explore the potential for these plasmid encoded cytokines to deviate the serum immunoglobulin profile we co-administered two different doses of these plasmids (10 and 50 μ g) with an optimal dose of pSLRSV.SgD (50 μ g). Figs. 4.7 A and B summarize mean serum isotype levels in mice immunized with 50.0 μ g pSLRSV.SgD co-administered with 10.0 or 50.0 μ g of a null plasmid (NS-60, NS-100), or plasmids encoding murine GM-CSF (GS-60, GS-100) or IFN γ (IS-60, IS-100). Mean serum isotype levels of IgG₁ and IgG_{2a} do show that a single dose of 10 or 50 μ g of pSLRSV.GM-CSF enhanced the amounts of serum IgG₁ (relative to serum IgG_{2a} levels) as compared to groups of mice immunized with pSLRSV.SgD + pSLRSV. Nul (NS-60 and NS-100), (Fig. 4.7 A). However, only the 10 μ g dose of pSLRSV.GM-CSF showed a predominance of IgG₁ in all 10 mice (Table 4.7). We anticipated, based on

isotype data presented in Fig. 4.4, and current literature, that mice immunized with pSLRSV.SgD + pSLRSV.IFN γ would show a tendency towards greater serum levels of IgG_{2a}. This did occur to some extent (Figs. 4.7 A and B), however, only 5 of 8, and 6 of 8 seropositive mice displayed a predominance of serum IgG_{2a} at 4 and 6 weeks, respectively (Table 4.7). Similarly, mice immunized with the 10 μ g dose of pSLRSV.IFN γ show an IgG₁:IgG_{2a} ratio of 1.23 and 1.08 before and after boosting, respectively. Once again the isotype switch to IgG_{2a} in individual mice immunized with 10 μ g of pSLRSV.IFN γ + pSLRSV.SgD (50 μ g) with 2 of 8 and 4 of 7 seropositive mice showing a predominance of serum IgG_{2a} before and after boosting, respectively (Table 4.7). One of the most surprising results involved the apparent pre- to post-boost isotype switch in mice immunized with 50 μ g of pSLRSV.GM-CSF (Figs. 4.7 A and B). The preboost isotype profile showed 9 of 10 seropositive mice with a serum predominance of gD specific serum IgG₁, while postboost sera showed 7 of 10 mice with a predominance of serum IgG_{2a}.

4.3.6 Co-administration of pSLRSV.GM-CSF with pSLRSV.SgD upregulates IL-4.

Co-administration of 10 μ g of plasmids encoding GM-CSF with a suboptimal dose of pSLRSV.SgD (10 μ g) enhanced the kinetics, and efficacy, of seroconversion and the magnitude of serum antibody levels (Fig. 4.3 and Table 4.4). We also demonstrated that co-administering equal doses of plasmid encoding IFN γ and the secreted form of BHV-1 gD increased the levels of serum IgG_{2a} (Fig 4.4 and Table 4.5). To further characterize the immune responses following co-administration of these two plasmid

Fig. 4.7 Relative amounts of IgG₁ and IgG_{2a} in mice immunized with plasmids encoding SgD and cytokines. Groups are as described in Fig. 4.6. Values are presented as optical densities (405 nm) following development of ELISA plates in the presence of the substrate p-nitrophenyl phosphate. All values were determined at a serum dilution of 1/100 and for mice 4 weeks (**Fig. A** -preboost) or 6 weeks (**Fig. B** -postboost) after the primary immunization. Mean values were calculated from optical densities determined for 7 to 10 individual mice. X-axis groups are as described in Fig. 4.6.

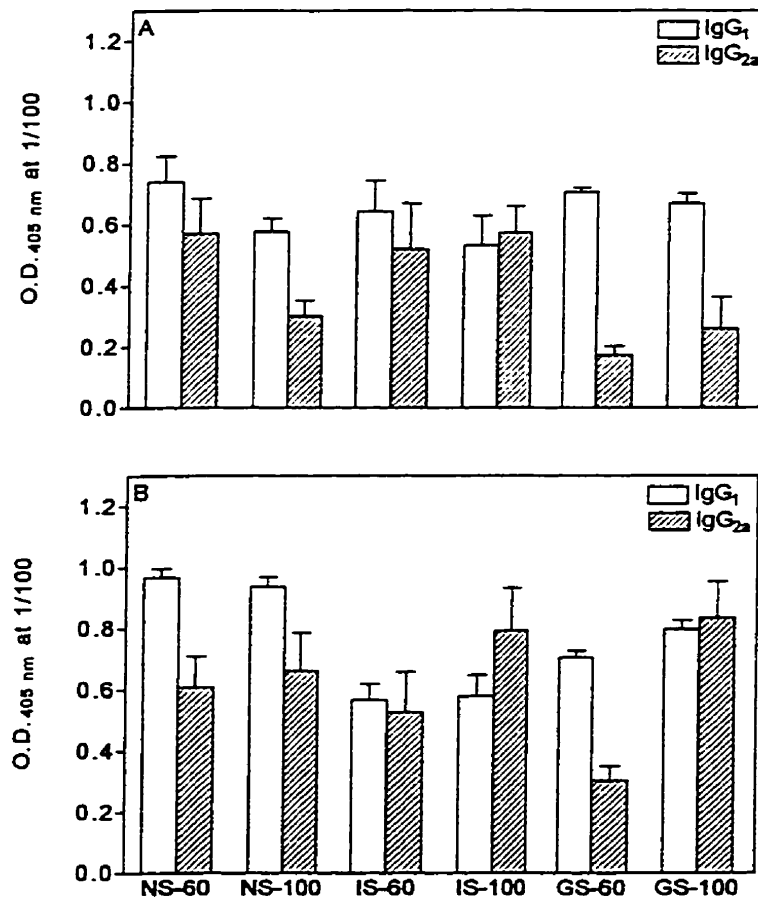


Table 4.7 Serum IgG₁ and IgG_{2a} levels after co-administration of pSLRSV.SgD with pSLRSV.GM-CSF or pSLRSV.IFN γ

Vaccine ^a Groups	# Mice With ^b Predominance of Serum IgG ₁	Mean Isotype O.D. (1/100) ^c		Ratio of IgG ₁ :IgG _{2a}
		IgG ₁	IgG _{2a}	
NS-60	8/9	.9664 ± .0294	.6076 ± .1027	1.59
IS-60	3/7*	.5663 ± .0514	.5253 ± .1332	1.08
GS-60	10/10	.7049 ± 0.229	.3023 ± .0462	2.33
NS-100	8/10	.9366 ± .0324	.6622 ± .1237	1.41
IS-100	2/8**	.5791 ± .0685	.7930 ± .1398	0.73
GS-100	3/10	.7981 ± .0285	.8363 ± .1185	0.95

^a Vaccine group designations are indicated in the legend to Fig. 4.6.

^b Levels of immunoglobulin isotypes were determined by ELISA as described in Methods (section 4.2.9). Data refers to the number of mice showing a predominance, not exclusivity, of serum IgG₁ for all seropositive mice at 6 weeks. Predominance was determined by the magnitude of O.D. _{405 nm} values for IgG₁ and IgG_{2a} at a 1/100 dilution.

^c Data is represented as the mean of O.D. _{405 nm} values for IgG₁ or IgG_{2a} in all seropositive mice.

* 4 mice from this group showed virtually identical serum O.D. values for both IgG isotypes

** 1 mouse from this group showed similar serum O.D. values for both IgG₁ and IgG_{2a}

encoded cytokines with pSLRSV.SgD, we assessed antibody isotype and cytokine secreting cell numbers within the spleens and draining lymph nodes from immunized mice (Tables 4.8 A and B). Because iliac node cell population recovery is limited to 10-15 million cells per mouse we were forced to pool samples from several mice in order to acquire enough cells for both the cytokine and immunoglobulin isotype ELISPOT assay (Tables 4.8 A and B). We pooled splenic and iliac lymph node cell populations based on serum isotype predominance in an effort to assess if serum isotype correlated to

Table 4.8 (A) Cytokine and antibody isotype profiles in mice showing dominance of serum IgG₁.

Vaccine ^a Groups	No. ^b of Mice	Cytokine ELISPOT ^c				Isotype ELISPOT ^d	
		Spleen		Iliac Node ^e		Iliac Node	
		IFN γ	IL-4	IFN γ	IL-4	IgG ₁	IgG _{2a}
pSLRSV.Nul	-	33 \pm 3	3 \pm 0	29 \pm 16	0	0	0
Saline	-	30 \pm 2	4 \pm 2	29 \pm 4	0	0	0
NS-60	8/9	101 \pm 15	13 \pm 4	86 \pm 2	32 \pm 12	53 \pm 16	12 \pm 3
IS-60	3/7	77 \pm 4	21 \pm 7	26 \pm 1	5 \pm 2	12 \pm 8	1 \pm 0
GS-60	10/10	195 \pm 7	103 \pm 6	99 \pm 7	175 \pm 17	24 \pm 3	9 \pm 1
NS-100	8/10	62 \pm 13	8 \pm 5	61 \pm 2	9 \pm 4	33 \pm 9	9 \pm 3
IS-100	2/8	120 \pm 8	19 \pm 2	n.d.	n.d.	8 \pm 2	4 \pm 1
GS-100	3/10	140 \pm 23	78 \pm 7	153 \pm 6	188 \pm 8	62 \pm 4	4 \pm 1

^a Vaccine group designations are indicated in the legend to Fig. 4.6.

^b No of mice showing a predominance of IgG₁ of the total number of seropositive mice (see Table 4.7).

^c Values are determined by taking the mean, \pm the standard deviation, of counts from triplicate wells

^d Values are calculated as in ^c

^e Is the primary draining lymph node of the deep tissues of the murine thigh (section 3.3.5).

n.d. - not done because too few cells harvested from the iliac node

Table 4.8 (B) Cytokine and antibody isotype profiles in mice showing dominance of serum IgG_{2a}.

Vaccine Groups	No. of Mice	Cytokine ELISPOT				Isotype ELISPOT	
		Spleen		Iliac Node		Iliac Node	
		IFN γ	IL-4	IFN γ	IL-4	IgG ₁	IgG _{2a}
NS-60	1/9	50 \pm 10	11 \pm 2	n.d.	n.d.	22 \pm 2	4 \pm 4
IS-60	4/7	71 \pm 11	24 \pm 5	34 \pm 3	10 \pm 1	19 \pm 1	16 \pm 4
GS-60	0/10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NS-100	2/10	291 \pm 31	27 \pm 6	n.d.	n.d.	53 \pm 6	44 \pm 4
IS-100	6/8	87 \pm 6	17 \pm 3	42 \pm 18	22 \pm 4	26 \pm 6	35 \pm 6
GS-100	7/10	219 \pm 17	235 \pm 11	n/a	n/a	n/a	n/a

^a Vaccine group designations are indicated in the legend to Fig. 4.6.

^b No of mice showing a predominance of IgG_{2a} of the total number of seropositive mice (see Table 4.7).

^c Values are determined by taking the mean, \pm the standard deviation, of counts from triplicate wells

^d Values are calculated as in ^c

^e Is the primary draining lymph node of the deep tissues if the murine thigh (section 3.3.5).

n.d.- not done because too few cells harvested from the iliac node

n/a - cells harvested from iliac node were dead as determined by Trypan blue exclusion

the IL-4, IFN γ , IgG₁ or IgG_{2a} ELISPOT pattern within specific lymphoid compartments. Co-administration of pSLRSV.GM-CSF leads to a substantial increase in IFN γ and IL-4 secretion in both the spleen and draining lymph node in a dose dependent manner (see GS-60 and GS-100 groups in Tables 4.8 A and B).

Data from pSLRSV.IFN γ co-administered with pSLRSV.SgD was difficult to interpret, largely due to insufficient numbers of cells from draining lymph nodes in the NS-60 and NS-100 control groups (Table 4.8 B). However, B-cell isotype ELISPOT data does suggest that increased numbers of IgG_{2a} secreting cells are apparent in mice

immunized with pSLRSV.IFN γ + pSLRSV.SgD, as compared to NS-60 and NS-100 control groups (Table 4.8 B). However, a higher dose of plasmids encoding murine IFN γ does not enhance the efficiency at which mice develop higher levels of IgG_{2a} in sera.

4.4. Discussion

Preliminary evidence demonstrated the immune modulatory roles for several plasmid encoded cytokines co-administered with a previously characterized DNA-based vaccine encoding a secreted form of the protective immunogen from bovine herpesvirus-1 glycoprotein D (Figs. 4.3, 4.4, 4.6 and 4.7, Tables 4.4, 4.5, 4.7 and 4.8). Despite the limited dose range assessed we noted that murine cytokines GM-CSF, IL-4, TNF α , IL-6 and IFN γ had measurable modulatory effects on immune responses following i.m. injection of C3H/HeN (H-2^k) mice with plasmids encoding these cytokines co-administered with plasmids encoding the secreted form of BHV-1 gD. Within the criteria established for assessment of immune modulation, co-administration of plasmids encoding IL-1 α , IL-2 and IL-10 with plasmids encoding the secreted form of BHV-1 gD, appeared to have little positive impact on the immunological outcome following immunization. Whether the absence of responses with these three cytokines reflects the single, low dose, assessed in the initial experiment (Fig. 4.3 and Table 4.4) requires further investigation. Thus, the immune responses to these three cytokines will not be discussed at this time. The focus of the discussion will include the possible mechanistic avenues associated with observed immunomodulatory outcomes following co-

administration of plasmids encoding GM-CSF, IL-4, IL-6, TNF α and IFN γ .

Serum antibody and cytokine profiles in C3H/HeN mice following immunization with three different doses of pSLRSV.SgD confirmed earlier studies which showed that animals with measurable serum anti-gD titers displayed a mixed IgG₁/IgG_{2a} isotype, with a predominance of IgG₁, and IFN γ secreting cells in the spleen (section 3.3.3, 3.3.4, 3.3.6 and Fig. 4.2). Despite the prevalence of IFN γ in the spleens of immune animals, serum levels of IgG₁ were typically 2 to 3 times greater, on average, than levels of IgG_{2a}. It was also demonstrated that a single dose of at least 50 μ g of pSLRSV.SgD was sufficient to elicit excellent serum IgG titres in 100 % of animals at 4 weeks post-immunization (Table 4.2). We found that a single dose of 10 μ g of this plasmid was suboptimal in terms of seroconversion efficacy, although the serum immunoglobulin subclass character and splenic cytokine profile was essentially identical to that observed for animals receiving the higher 50 μ g dose (Table 4.2 and 4.3, Fig. 4.4).

Initial experiments with plasmids encoding cytokines were essentially a prescreen for cytokines that might possess an immunomodulatory function. On the understanding that seroconversion efficacy, magnitude, and character of serum immunoglobulin profiles, were of utmost importance in controlling many infectious viral diseases, we established these as criteria for determination of immune modulation by co-administered plasmid encoded cytokines. We clearly showed that co-administration of plasmids encoding the murine cytokine GM-CSF enhanced early serum antibody magnitude and seroconversion efficiency when utilized in the context of suboptimal doses (10 μ g) of pSLRSV.SgD (Fig. 4.3). Several articles have been published recently describing the immunological

outcome to DNA-based vaccines co-administered with plasmids encoding GM-CSF and in most instances enhancement has been demonstrated (Xiang and Ertl, 1995; Syrengelas et al., 1996; Conry et al., 1996a; Geissler et al., 1997; Iwasaki et al., 1997; Kim et al., 1997). Xiang and Ertl (1995) first described enhancement of early, post-immunization, viral neutralizing titres and post-boost survival, following challenge, in C3H/He mice immunized with plasmids encoding rabies virus G protein co-administered with plasmids encoding GM-CSF. They found that the efficacy of protection and the magnitude of serum antibody titer correlated directly with the dose of plasmids encoding GM-CSF to a maximum of 250 µg. In this particular study, mice were immunized initially with a mixture of plasmids encoding both G protein and GM-CSF. All animals were subsequently boosted at 2 or 3 weeks with plasmids encoding G protein only. Cytokine profiles from *in vitro*, antigen stimulated, splenocytes harvested 2 weeks post-boost indicated that IL-2, IL-3, and GM-CSF secretion were upregulated in animals receiving plasmids encoding GM-CSF, as compared to animals immunized with plasmids encoding G protein alone. In this instance, levels of splenic IL-4 and IFN γ were not assessed. Iwasaki et. al. (1997), demonstrated that i.m. immunization of BALB/c (H-2^d) mice with a mixture of plasmids expressing a non-immunogenic mutant of influenza nucleoprotein (NP) and GM-CSF resulted in significant enhancement of cytolytic activity only after boosting. These authors co-administered 100 µg of each plasmid and boosted with both plasmids at 3 and 6 weeks. Conversely, Kim et. al. (1997) were not able to demonstrate enhancement of CTL activity in BALB/c mice when plasmid encoded GM-CSF was co-administered with plasmid encoding several antigens (Env, Gag/pol, Vif and Nef) from

human immunodeficiency virus (HIV)-1. However, co-administration of plasmids encoding GM-CSF with plasmids encoding Env and Gag/pol showed enhancement of antigen-specific serum immunoglobulin and splenic lymphocyte proliferation following a single immunization with 50 µg of each plasmid, or following boosting at day 14. Geissler et. al. (1997) demonstrated that BALB/c mice, pretreated with a 0.9 % bupivacaine injection i.m., receiving a multi-site immunization with 50 µg of plasmids encoding GM-CSF and the nonsecreted Hepatitis C virus core protein displayed increased seroconversion efficacy with a moderate increase in CTL activity. It should be pointed out that Geissler et. al. (1997) utilized an injection protocol that involved boosting with plasmid encoded core protein in the opposite leg from which the initial cytokine/antigen vaccination occurred. Conry et. al. (1996) demonstrated that immune responses in C57BL/6 (H-2^b) mice, following ballistic delivery of 2 µg of plasmids encoded a carcinoembryonic antigen (CEA) to the skin, was only enhanced by plasmids encoding GM-CSF if delivery of this cytokine preceded delivery of CEA to the same site by several days. Indeed simultaneous ballistic delivery of these two plasmids failed to augment humoral or cell-mediated, CEA specific, immune responses. In this instance the authors included a reporter construct as a control plasmid and showed that pre-administration of this DNA prior to delivery of CEA resulted in at least 60% of mice showing some augmentation of serum antibody responses. Finally, Syrengelas et. al. (1996) demonstrated that plasmids encoding an in-frame genetic fusion of GM-CSF to the humanized mouse idiotype determinant of B-cell lymphoma, delivered i.m. or i.d. into C3H/HeN mice, enhanced early anti-idiotype serum titers. It may also be of some

interest to note that i.m. delivery of 100 µg of plasmids encoding this idiotypic resulted in significantly more serum IgG_{2a} in mice than following i.d. immunization.

It is apparent that GM-CSF plays a significant and varied role in the enhancement of immune responses to co-administered DNA-based vaccines. The specific mechanism of immune modulation of this cytokines *in vivo* remains to be established. However, it seems reasonable to hypothesize that seroconversion enhancement facilitated by GM-CSF is the result of a specific action on resident dendritic cells. It has been demonstrated that GM-CSF plays a critical role in recruitment and maintenance of the ability to capture and process antigen in human and mouse dendritic cells (Heufler et al., 1987; Inaba et al., 1992; Sallusto and Lanzavecchia, 1994; Lutz et al., 1996). This cytokine has also been demonstrated to upregulate antigen acquisition and presentation functions in bone marrow derived macrophages (Fischer et al., 1988). This information, and the ELISPOT cytokine data presented in Tables 4.8 A and B, support the hypothesis that secretion of GM-CSF by cells harboring plasmids encoding this cytokine are directly responsible for the significant enhancement of seroconversion efficacy and indirectly responsible for enhancement in the magnitude of mean serum immunoglobulin titers. It would seem plausible to predict that the increase in local recruitment of immature, antigen-uptake proficient, dendritic cells, and local maintenance of antigen capture function, is responsible for enhancing the number of responders regardless of variations in animal immunoresponsiveness and variability in vaccination technique. An alternative explanation, in light of recent evidence that professional antigen presenting cells at the injection site are capable of naked DNA uptake (Raz et al., 1994; Condon et al., 1996), is

that dendritic cells transfected with plasmids encoding GM-CSF may traffic to the local draining lymph node and create a microenvironment conducive to the enhancement of antigen-specific T-, and B-cell numbers.

Enhancement of antigen-specific serum immunoglobulin levels in mice immunized with plasmids encoding IL-4, co-administered with pSLRSV.SgD, without the concurrent increase in the number of responders, as observed in mice receiving plasmid encoded GM-CSF, supports suggestions that GM-CSF functions directly to enhance vaccine efficacy and indirectly, via induction of IL-4 in draining lymph nodes, to enhance the magnitude of serum IgG titers. Conversely, although IL-4 has been demonstrated to play a role in upregulation and maintenance of the antigen capture function of dendritic cells, it seems reasonable to suggest that at the single dose tested (10 µg) this function of IL-4 is minimized and that enhancement of serum titer is facilitated by a mechanism distinct from that induced by GM-CSF (Fig. 4.3 and Table 4.4), (Sallusto and Lanzavecchia, 1994; Lutz et al., 1996). Interleukin- 4 has also been demonstrated to play a key role in the immunoglobulin switch to IgG₁ and IgE (Snapper and Mond, 1993), the induction of Th2-type T-cells, inhibition of nascent Th1 phenotype and deviation of short-cultured, antigen-specific, Th1 T-cells towards a Th2 phenotype (Rocken et al., 1996). IL-4 has also been shown to augment B-cell antibody synthesis through upregulation of surface IgM and CD40 (Gordon et al., 1988; Shields et al., 1989), in the generation of CTLs and in the recruitment of activated macrophages (Trenn et al., 1988; Golumbeck et al., 1991; Banchereau, 1991a). Despite early evidence that IL-4 can enhance the development of antigen-specific CTLs, a recent paper in which BALB/c mice

were immunized with 50 µg of plasmid encoded IL-4, co-administered with 50 µg of plasmid encoded Hepatitis C virus core protein, failed to demonstrate enhancement of T-cell mediated cytotoxic activity (Geissler et al., 1997). Although CTL activity was not enhanced in this instance, seroconversion efficiency was augmented significantly with a slight increase in mean serum IgG titers. Geissler *et. al.* (1997) did not assess serum immunoglobulin subclass, however, they did determine that the splenic cytokine profile from plasmid encoded IL-4 and HCV immunized mice, was characteristic of a Th0 phenotype upon *in vitro* restimulation.

It has been demonstrated that the induction of antigen-specific Th2-type cells requires simultaneous exposure of uncommitted T-cells to IL-4 and TCR engagement by APC MHC II/peptide complexes (Rocken et al., 1996). Enhancement of mean serum titers without a substantial enhancement in levels of serum IgG₁ (Table 4.5) suggests that the dose of IL-4 was sufficient to directly, or indirectly, augment initial B-cell interaction with antigen or subsequent interactions with antigen associated with follicular dendritic cells, but insufficient to facilitate a deviation towards greater levels of Th2. Selective increases in serum IgG₁ levels have been demonstrated after 3 i.m. injections of very high doses (100 µg each) of plasmids encoding IL-4 and subsequent immunization with transferrin formulated in aluminum hydroxide (Alum), (Raz et al., 1993). Direct action of IL-4 on B-cells could occur by several mechanisms: IL-4 derived from plasmids taken up and expressed by cells at the injection site, expression within the draining lymph node by interstitial dendritic cells transfected *in vivo* at the injection site prior to maturation and mobilization, or by cells within the draining lymph node

transfected by the wave of plasmid DNA that washes through draining lymphatics and lymph nodes immediately following injection (section 3.3.5). It may also be possible that recruitment of short term Th1-type T-cells and subsequent exposure to injection site microenvironmental IL-4 and antigen may lead to deviation towards greater numbers of Th2 cells and augmentation of B-cell responses upon movement into draining lymph nodes (Rocken et al., 1992). However, in the absence of cytokine ELISPOT data it is difficult to ascertain if the enhanced levels of serum IgG, observed in mice immunized with plasmids encoding IL-4 co-administered with pSLRSV.SgD, is occurring by a mechanism other than deviation towards greater numbers of antigen-specific Th2-type CD4⁺ T-cells (Davis et al., 1997; Levitsky, 1997). Also, it has been demonstrated that IL-4 can significantly enhance proliferative capacity of B-cell receptor (BCR) or CD40 engaged B-cells and induce lipopolysaccharide (LPS) stimulated B-cells to produce IgG₁ and IgE (Alderson et al., 1987; Defrance et al., 1987; Snapper and Paul, 1987; Banchereau et al., 1991b). However, there is some recent controversy as to whether antigen-specific B-cell responses are indeed upregulated by IL-4 (Taylor, 1995).

Mechanistic details regarding the ability of TNF α to augment immunity *in vivo* have not been worked out. However, there is clear evidence that T-dependent antigen-specific B-cell responses are enhanced by co-administration of doses well below the dose range demonstrated to cause cachexia and tissue toxicity (Ghiara et al., 1987; Schijns et al., 1994). Intraperitoneal infusion of recombinant TNF α , at doses as low as 1.3 ng/mouse, enhanced antigen-specific B-cell responses against sheep red blood cells and rabies virus, respectively. Despite evidence in the literature that exposure of cultured dendritic cells to

recombinant TNF α leads to a 10 fold decrease in presentation of soluble antigen and a 100 fold decrease in the capacity to present immunoglobulin antigen complexes, we have clear evidence that TNF α can enhance the early humoral response (Fig. 4.3 and Table 4.4), (Sallusto and Lanzavecchia, 1994). It has been suggested that the dose, as we discovered with GM-CSF and IFN γ , may be a pivotal issue in the outcome of experiments where TNF α deviation of T-dependent immune responses are studied. Indeed, Sallusto and Lanzavecchia (1994) hypothesized that too much TNF α may lead to a local or generalized immunosuppressive event as a result of widespread maturation and mobilization of peripheral dendritic cell populations. One might predict that smaller doses of TNF α may enhance mobilization and maturation without an acute compromise of antigen presentation. Our data suggests that a relatively low dose of TNF α modulates the immune responses to pSLRSV.SgD in two distinct ways. Co-administration of plasmids encoding TNF α appears to enhance the early kinetics of serum IgG response in a manner distinct from that observed in the GM-CSF group, and also deviates the normal serum immunoglobulin isotype responses towards greater levels of IgG_{2a}. The observed increases in levels of serum IgG_{2a} are inconsistent in mice within this group and suggest that the dose required to drive immunoglobulin switch to IgG_{2a} is suboptimal, or that TNF α must synergize with other cytokines to deviate the immunoglobulin isotype in a more efficient manner. This observation is of some importance because to our knowledge TNF α has not been correlated to a specific deviation of serum immunoglobulin towards IgG_{2a}. However, administration of TNF α with IL-12 has been demonstrated to enhance the levels of Th1-type T-cells in mice (Ahlers et al., 1997).

Certainly, TNF α has been demonstrated to upregulate IFN γ from NK cells (Scharton and Scott, 1993), however, it is unknown if this mechanism explains the apparent immune deviation observed. It has been demonstrated that serum levels of IgG_{2a} and IgG₁ were elevated when endogenous NK cells were activated by poly (I:C) (Wilder et al., 1996), and that only the IgG_{2a} increases were NK cell dependent. Recently the adjuvant capacity of certain hypomethylated CpG motifs, within bacterially derived DNA, were shown to be capable of inducing NK cells to secrete significant amounts of IFN γ , however, serum isotype data from the null/pSLRSV.SgD control group suggests that immunostimulatory sequences within this plasmid DNA are not responsible for increased levels of IgG_{2a} observed in the TNF α group. (Klinman et al., 1996).

TNF α has been shown to be of critical importance in the promotion of the follicular dendritic meshwork within developing germinal centers (Goodnow, 1997). TNF α knockout, and TNFR-1^{-/-}, mice lack lymph nodes, the majority of Peyer's patches, and lack follicles within the spleen (Pasparakis et al., 1996; Kosco-Vilbois et al., 1997). It is hypothesized that CXCR4 and BLR1 chemokine receptors serve as important facilitators for B-cell entry into follicular niches which are then induced to produce TNF α (Goodnow, 1997). It is possible that plasmids encoding TNF α serve as an exogenous source of cytokine to enhance the early kinetics of germinal center development or number.

IL-6 is a multifunctional cytokine that is produced by both lymphoid and nonlymphoid cells and plays a key role in acute phase and immune responses as well as haematopoiesis (Hirano et al., 1990). This cytokine is believed to play an important role

in B-blast development, and to induce synthesis of secretory type IgG. It also appears to augment the development of monocytes into mature tissue macrophages. We demonstrated that co-administration of plasmids encoding IL-6 with pSLRSV.SgD has little effect on two week mean titers or seroconversion efficiency, however, 4 week mean serum titers were essentially equivalent to those observed for IL-4, GM-CSF and VSA3/tgD (Fig. 4.3 B and Table 4.4). IL-6 has been demonstrated to augment both primary and anamnestic antigen-specific humoral responses, although, it appears that IL-6 may play a more prominent role in the anamnestic responses (Takatsuki et al., 1988). It was demonstrated that macrophages and CD4⁺ T-cells were critical in the immunological enhancement observed upon injection of recombinant IL-6. Recently it has been suggested that IL-6 may be the initiating signal that deviates the primary immune response toward a Th2-type response (Rincon et al., 1997). Despite recent evidence suggesting that CD3⁺CD4⁺ NK1.1⁺ T-cells are a potential early source of IL-4, it has been shown that these cells are also capable of producing IFN γ (Yoshimoto and Paul, 1994; Vicario and Zlotnik, 1996) which, with IL-12, has been demonstrated to be involved in the deviation of the immune response toward a Th1 type phenotype (Gajewski and Fitch, 1988; Scott, 1991). Although the activation signals for NK1.1⁺ T-cells have not been definitively identified there is evidence that signalling is mediated through surface engagement of the MHC-like molecule CD1 on antigen presenting cells resulting in upregulation of cytokine secretion from this population of T-cells (Yoshimoto et al., 1995). Despite this evidence, Rincon et al. (1997) put forth an alternative hypothesis for the indirect involvement of IL-6 in the induction of the Th2

phenotype that involves the polarization of naive CD4⁺ T-cells into effector cells by the induction of endogenous IL-4, that originates from naive CD4⁺ T-cells, and acts in an autocrine or paracrine fashion. Significant levels of IL-6R α have been identified on unstimulated CD4⁺ T-cells and 100 fold increases in T-cell IL-4 mRNA levels have been demonstrated upon antigen stimulation in the presence of exogenous IL-6 or IL-4. A second possible explanation for the apparent ability of IL-6 to enhance 4 week antigen-specific serum titers was determined using IL-6 knockout mice (Kopf et al., 1994). These authors noted that IL-6 deficient mice showed a 5-10 fold reduction of T-cell dependent Ab responses against Vesicular Stomatitis virus (VSV). Transgenic mice overexpressing IL-6 developed a severe plasmacytosis and accompanying increase in polyclonal serum IgG₁. Although IL-6 production in germinal centers has been demonstrated, it is unknown whether the apparent augmentation of plasma blast expansion occurs directly or by the hypothesized initial expansion of IL-4 producing T-cells suggested by Rincon et. al. (1997). Whatever the mechanism, the kinetic differences between humoral modulation observed in IL-6 co-injected mice and mice receiving GM-CSF, TNF α and IL-4 suggest a mechanism that is unique to IL-6.

Interferon γ (IFN γ) is produced predominantly by T-cells of the Th0, Th1 and Tc1-type phenotypes, as well as, activated NK cells (Mosmann and Coffman, 1987; Scharton and Scott, 1993). Secretion of IFN γ , in conjunction with IL-2 and Lymphotoxin (LT) is felt to typify activated T-cells of the Th1 phenotype. This cytokine is multifunctional and has been described to: enhance presentation of antigen by inducing upregulation of MHC I/II molecules (Steeg et al., 1982), inhibit immune deviation toward the Th2 phenotype

(Gajewski and Fitch, 1988), and selectively enhance the immunoglobulin switch to the IgG_{2a} isotype (Snapper and Paul, 1987). We had previously demonstrated that i.m. injection of plasmids encoding the secreted form of BHV-1 gD resulted in a splenic cytokine profile indistinguishable from that observed in mice immunized with membrane anchored or cytosolic version of BHV-1 gD (section 3.3.1). However, draining lymph node cytokine profiles, at 6 weeks post-immunization, showed elevated recall levels of IL-4 relative to the authentic and cytosolic versions of gD as well as serum isotype profiles that were predominantly, but not exclusively, IgG₁ (section 3.3.6). Conversely, mice immunized with plasmids encoding the authentic, membrane-associated BHV-1 gD and the cytosolic version of gD displayed serum isotype profiles that were predominantly IgG_{2a} and appeared to be the result of the development of IgG_{2a} specific antibody secreting cells within the draining lymph node (sections 3.3.3 and 3.3.6). Co-administration of plasmids encoding IFN γ with plasmids encoding a secreted form of BHV-1 gD (pSLRSV.SgD) enhanced mean serum IgG_{2a} levels (Fig. 4.4 and Table 4.5). However, this response was inconsistent in individual animals. This inconsistency did not appear to be a dose related phenomenon and is, perhaps, not surprising in light of evidence that has demonstrated that IFN γ receptor knockout mice can still produce some IgG_{2a} and IgG3 (Huang et al., 1993). Certainly recent evidence suggests that immunoglobulin class switching is a phenomenon that relies on multiple pathways and does not rely, exclusively, on cytokine switch factors such as IFN γ or IL-4 (Snapper and Mond, 1993; Coffman and von der Weid, 1997; Sangster et al., 1997). This hypothesized immunological redundancy involvement in isotype switching to IgG_{2a}, in the face of an

antigen that promotes IgG₁, may explain why some mice failed to display a predominance of serum IgG_{2a}.

Finally, we demonstrated that low doses of plasmids encoding IFN γ co-administered with plasmids encoding the secreted form of BHV-1 gD, suppressed the magnitude of mean serum antibody levels while high doses of plasmids encoding this cytokine enhanced primary and anamnestic humoral responses (Figs. 4.3 and 4.6). Suppression of both T-, and B-cell responses in mice immunized with plasmids encoding rabies virus G protein co-inoculated with plasmids encoding murine IFN γ have been described (Xiang and Ertl, 1995). It may be important to note that these authors used the relatively weak SV40 early promoter to drive expression of this cytokine and utilized a single dose of 50 μ g of this plasmid. We noted that, with a stronger expression cassette, 10 μ g of pSLRSV.IFN γ suppressed humoral and draining lymph node cytokine responses (Fig. 4.6 and Table 4.8 A). In an effort to explain depressed immune responses in their model Xiang and Ertl (1995), suggested that the upregulation of MHC antigens on the normally quiescent population of myocytes would result in increased susceptibility of antigen expressing myocytes to antigen-specific cytotoxicity thereby limiting the amount of antigen available for capture, processing and presentation (Hohlfeld and Engel, 1994). It is also plausible that IFN γ mediated reduction in antigen uptake by local dendritic cells, regardless of enhanced presentation capacity, may result in premature migration of mature dendritic cells to the draining lymph nodes prior to optimal uptake of local antigen ((Kitajima et al., 1996).

There is an increasing awareness that the dose of cytokine(s), and cellular

microenvironment in which they occur, can play a significant role in modulating immune responses (Kishimoto et. al., 1994; Hughes and Babiuk, 1992; Finkelman and Maliszewski, 1997; Paul and Seder, 1994). While recognizing that this is the case it must be understood that quantifying the expression level of plasmid encoded cytokines *in vivo* is a significant and challenging undertaking beyond the scope of this thesis. However, in an attempt to address this issue many researchers involved in DNA-based vaccine research are varying the dose of plasmids encoding cytokines in an effort to better understand the roles these cytokines can play *in vivo*. We have demonstrated that the dose of co-administered plasmids encoding either GM-CSF or IFN γ with plasmids encoding a secreted version of BHV-1 gD, can have vastly different effects on the immunological outcome. Whether this is due to different levels of expression directly related to increased plasmid concentration or due to other factors, such as the quantity of immunostimulatory sequences present in the plasmids, is in need of clarification. We have demonstrated that GM-CSF affects humoral and/or immunological responsiveness, at 2 weeks post immunization, regardless of the dose co-administered. However, it is clearly evident that high doses of plasmids encoding GM-CSF, in the absence of boosting, can have a suppressive or deviating effect such that the magnitude of the humoral response is significantly reduced. Conversely, priming and boosting with high doses of plasmids encoding GM-CSF significantly enhanced antigen-specific titers, and substantially increased the levels of serum IgG_{2a}. Co-administration of plasmids encoding GM-CSF strongly upregulated antigen-specific IL-4 secretion in both the spleen and the draining lymph nodes of immunized mice. This, in part, may explain the

observed enhancement of serum immunoglobulins and IgG₁ titers for low doses of plasmids encoding GM-CSF. Plasmids encoding murine IFN γ co-administered with plasmids encoding a secreted version of BHV-1 gD also displayed dose dependent effects. Mice immunized with a low (10 μ g) dose of plasmids encoding IFN γ displayed either suppression of immunity or a polarization towards a Th1-type immune phenotype. However, mice immunized with a high dose (50 μ g) of this construct developed excellent pre-boost titers and displayed a 2-fold increase of serum titers following a boost. We also demonstrated that co-administration of low and high doses of plasmids encoding IFN γ with plasmids encoding the secreted form of BHV-1 gD elevated serum isotype levels of IgG_{2a}, although the efficacy of this switch was inconsistent on an individual mouse basis. Several other observations were made and included: evidence of plasmid uptake competition *in vivo*, immune deviation towards elevated levels of serum IgG_{2a} by co-administration of plasmids encoding TNF α , evidence that co-administration of plasmids encoding IL-4, IL-6 as well as TNF α enhance mean serum IgG levels with differing kinetics. The failure of plasmids encoding IL-2 and IL-1 α to elicit measurable enhancement of responses to mice co-immunized with plasmids encoding a secreted form of BHV-1 gD may simply reflect the single dose assessed or the assays utilized to determine modulation.

5.0 IMMUNIZATION WITH A DNA-BASED VACCINE IN THE FACE OF PASSIVE ANTIBODY ELICITS CELL-MEDIATED AND ACTIVE HUMORAL RESPONSES

5.1 Introduction

One of the prevalent problems faced in vaccine development is the inability to elicit active humoral immunity and reproducible cell-mediated immunity in neonatal animals born to immune mothers (Harte et al., 1983; Bona and Bot, 1997). Two primary and distinct problems exist which hinder the development of protective immunity in neonates. The first problem describes the relative immaturity of the neonatal immune system and the inability of these animals to mount an effective immune response to the vaccine doses normally given to adult animals (Ridge et al., 1996; Sarzotti et al., 1996). In these instances, the relevance of mouse models assessing peripheral immune deficiencies in neonates is questionable when evidence demonstrates substantial differences between species in the degree of peripheral immune precociousness. Furthermore, the mechanism for observed transient deficient, or polarized, responses to T-dependent antigens may also differ greatly between species (Forsthuber et al., 1996). For example murine neonates display a profound reduction in lymphocyte numbers in peripheral lymphoid organs while humans and other mammalian neonates display substantial development of peripheral lymphoid organs during the neonatal period (Washington et al., 1994; Kimpton et al.,

1995; Ridge et al., 1996). However, human neonates show little CD40 ligand (CD154) expression on peripheral T-cells in the immediate neonatal period (Durandy et al., 1995). Interaction of this ligand with CD40 receptor on interstitial dendritic cells (IDC), B-cells and macrophages elicits a critical costimulatory signal to T-cells (Stuber et al., 1996). Porcine neonates also show a profound peripheral immunodeficiency for up to four weeks postnatally (Metzger et al., 1978). In light of the variety of mechanisms potentially involved in transient neonatal immunodeficiencies, as well as the degree of deficiency, it seems reasonable to identify a time point at which waning maternal antibodies are still protective and postnatal immune potential has begun to equal that observed in adults. Regardless, the potential for induction for high zone tolerance, or deviation towards a Th2 type immunity, in any given species must be taken into account prior to assessment of active immune responses to vaccination.

The second reason for failure of neonatal animals to respond efficiently to vaccination is the well documented, but poorly understood phenomenon where maternal antibody inhibits the development of active immunity (Xiang and Ertl, 1992). Although maternal antibody is absolutely critical for early protection against many neonatal pathogens, it leads to a situation where there is an unavoidable window of susceptibility to disease following the decline of maternally derived antibody and the development of active humoral responses (MacDonald, 1992). This window of susceptibility occurs because the maternally derived passive antibody titer capable of inhibiting the response to vaccination is frequently lower than the titer required for protection. Thus young animals must "wait" until suppressive passive antibody titers have declined to the point where

they are no longer able to interfere in the development of an active humoral response to vaccination. Thus the "window of susceptibility to disease" is determined from the time the maternal antibody titers wane to nonprotective levels to the point at which endogenously produced antibody titers become protective. This window can often amount to several weeks and, despite nomograms designed to calculate the point at which maternal titers have decayed, calculations can leave many young animals susceptible for days or weeks longer than necessary. Multiple immunizations have been utilized, with varying degrees of success, to avoid unnecessarily long periods of disease susceptibility. However, this approach may be more acceptable, and easier to implement in humans as opposed to many food animal species. To summarize, we have a spectrum of transient immunodeficiencies in the immediate neonatal period that appear to prevent or actively suppress the induction of cell-mediated immune responses to varying degrees depending on the species. To compound matters, maternal antibody provided by immune mothers specifically inhibits B-cell mediated immunity and indirectly impedes the expansion of a nascent population of primed T-cells. These two problems are interrelated but mechanistically distinct. However, a number of proposed features unique to immunization with DNA-based vaccines suggests this approach has some potential to possibly overcome deficiencies characteristic of neonatal animals.

Genetic immunization, variously termed as nucleic acid, polynucleotide, somatic transgene, or DNA-based vaccination was first described 5 years ago (Tang et al., 1992; Donnelly et al., 1993; Ertl and Xiang, 1996; Hassett and Lindsay Whitton, 1996; Ulmer et al., 1996b; Gerloni et al., 1997). This novel method of immunization involves the *in vivo*

introduction of plasmid encoded antigens under the control of eukaryotic regulatory elements such that cells at the site, and perhaps distal to the site, of plasmid introduction become transfected (Wolff et al., 1992; Raz et al., 1994). These transiently transfected cells then express antigen that is either directly or indirectly presented to naive T and B-cells of the host animal such that antigen-specific humoral and cell-mediated immunity results (Ulmer et al., 1996c). Mice immunized with DNA-based vaccines typically mount potent CTL responses, frequently accompanied by Th 1 type immune response and often with significant serum IgG titers. The mechanism involved in the development of immune responses following plasmid immunization is not completely understood. However, it is believed that *in situ* transfection of dendritic cells and the accompanying adjuvant effects of hypomethylated CpG motifs within the plasmid DNA play a significant, and perhaps, critical role in the efficient development of immunity to these novel vaccines (Pertmer et al., 1995; Condon et al., 1996; Pissetsky, 1996a; Carson and Raz, 1997; Roman et al., 1997). Early work with intramuscular (i.m.) delivery of DNA-based vaccines demonstrated that transfected plasmid DNA displayed the ability to persist extrachromosomally and that the immune response in animals immunized with these vaccines was highly durable, sometimes lasting the lifetime of the animal without the need for further boosting (Wolff et al., 1992; Davis et al., 1996). Once again the underlying mechanism for this apparent phenomenon was not understood. However, researchers felt that the observed durability may reflect the apparent persistence of plasmid DNA within the transfected cells and likelihood for prolonged expression. This seemed a reasonable hypothesis given that antigen persistence has been demonstrated to

be pivotal in ensuring long lasting durable humoral immunity (Bachman et al., 1996). However, recent evidence suggests persistence of expression is determined to a large extent by the intrinsic immunogenicity of the plasmid encoded antigen and possibly the cell type transfected *in situ* (Davis et al., 1997).

In the present study, we investigated the humoral and T helper responses in 6-8 week old C3H/HeN and C57BL/6 mice passively immunized with hyperimmune sera or antigen-specific monoclonal antibodies prior to immunization with a single dose of a DNA-based vaccine encoding a truncated version of the bovine herpesvirus-1 (BHV-1) glycoprotein D (Tikoo et al., 1993). This truncated version of BHV-1 gD produces an extracellularly secreted glycoprotein lacking the normal hydrophobic transmembrane anchor seen in the authentic viral glycoprotein. In choosing this 6-8 week old mouse model we eliminated the compounding problem of murine neonatal immunodeficiency and the propensity to tolerize or deviate responses towards Th2-type immunity. This allowed us the opportunity to study the effects of passive antibody titers on the immune responses to DNA vaccination, and to gain some insight on the potential for this vaccine approach to overcome maternal antibody suppression.

5.2 Materials and Methods

5.2.1 Mice.

Female 5-6 week old C3H/HeNCrIBR and C57BL/6 mice were purchased from Charles River (Canada). All mice were acclimated for 1-2 weeks prior to vaccination with DNA-based or conventional vaccines.

5.2.2 Plasmid Constructs.

All restriction enzymes and DNA modifying enzymes as well as markers and plasmids were purchased from Pharmacia or New England Biolabs unless indicated otherwise. Expression cassettes utilizing the immediate early promoter/enhancer regions from MCMV (Murine Cytomegalovirus) and RSV-LTR (Rous Sarcoma Virus -Long Terminal Repeat) were constructed (see section 3.2.1). These regulatory elements were placed in the high copy plasmid pSL301[™] (Invitrogen Corp., San Diego, CA) to generate the expression plasmids pMCEL.Nul and pSLRSV.Nul. Creation of an expression cassette expressing a secreted, or truncated, version of BHV-1 gD was described previously (Tikoo et al., 1993). Briefly, vector pRSV1.3gDt, which encodes a truncated form of gD, was generated by introduction of a unique *Xho*I site at the *Sac*II site immediately adjacent to the transmembrane domain of pRSV1.3 encoding the full length, or authentic, gene for BHV-1 gD. Following restriction digestion, and end repair, this unique *Xho*I site was blunt-end ligated to a short linker sequence encoding the unique restriction site *Nhe*I and three stop codons. Expression of this truncated gene leads to secretion of a glycosylated product approximately 62-kDa (Tikoo et al., 1993). Plasmids pSLRSV.AgD (AgD = authentic or full length gD) and pSLRSV.SgD (SgD = secreted or truncated gD), were created by ligating the 947-bp *Nde*I fragment from pRSV1.3 and ligating it into *Nde*I digested pMCEL.AgD and pMCEL.SgD (not shown). The *Nde*I fragment from pRSV1.3 contains the RSV-LTR promoter/ enhancer region and a portion of the 5' end of the gD gene. Ligation of a 585-bp *Nde*I-*Bgl*II fragment from pRSV1.3 ligated into *Nde*I-*Bgl*II digested pMCEL.Nul generated plasmid pSLRSV.Nul (Tikoo et

al., 1990; Tikoo et al., 1993). *In vitro* expression of all constructs was demonstrated by immunoprecipitation of truncated gD from media harvested following stable and transient transfection of L929 (ATCC No. NCTC clone 929) and COS-7 (ATCC No. CRL 1651) cell lines (data not shown).

5.2.3 Passive Immunization.

Hyperimmune donor mice (C3H/HeN) were prepared by i.m. immunization with 100 µg of pSLRSV.SgD. Seropositive mice were then boosted on days 14 and 24 with recombinant truncated gD (1.0 µg and 1.5 µg/mouse, respectively) emulsified in VSA3[™] adjuvant (Biostar, Saskatoon, Saskatchewan, Canada), (VSA3/rtgD). Sera, from hyperimmune donor mice, was harvested 10-14 days following the final boost.

All donor mouse hyperimmune blood was collected by cardiac puncture of halothane anaesthetized mice. Sera were prepared by brief centrifugation of pooled blood and transferred to sterile 15 mL centrifuge tubes (Corning Inc., Corning, N.Y.). Recipient mice were physically restrained and intraperitoneally (i.p.) injected with an appropriate amount of hyperimmune antisera to result in passive antibody serum titers in recipient mice of between 10,000 and 20,000 ELISA units. A formula to predict recipient mouse serum titers prior to passive transfer of hyperimmune sera was as follows: $x = a/b \times c$, where a = vol. hyperimmune sera to be injected i.p., b = estimate of extracellular fluid volume (approximately 20% of body weight in grams, or approximately 30% of the estimated total body water -60% of body weight) and c = the ELISA titer of pooled donor sera. All recipient mouse titers were verified after 24 hours using a gD-specific ELISA.

5.2.4 Immunization with Plasmid and Conventional Vaccines.

Inbred 7 week old, female C3H/HeN mice were injected with plasmid DNA purified by anion-exchange column (Qiagen Inc., Santa Clarita, CA) and dissolved in normal saline. All injections of DNA were i.m. while injections of purified antigen (authentic or recombinant truncated gD) formulated in either VSA3 (Biostar Inc.) or alum (Alhydrogel™, Cedarlane Laboratories Ltd., Hornby, Ontario) were subcutaneous (s.q.) at the dorsal midline of the thorax on each mouse. Mice immunized with DNA were physically restrained and injected using a 0.5 mL disposable Microject syringe with a 29 gauge needle. All mice received doses of plasmid DNA split between the left and right quadriceps muscle mass. Mice were not boosted unless indicated otherwise. Nonlethal tail bleeds were carried out every 2 weeks until the experiments were terminated. Mice were euthanized by an overdose of Halothane™ (MTC Pharmaceuticals, Cambridge, Ontario).

5.2.5 ELISA.

Immunolon 2 microtitre plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with purified recombinant truncated gD (Biostar Inc.) at a concentration of 0.050 µg/well. Antigen was diluted with ELISA Coating buffer (.012 M Na₂CO₃, .038 M NaHCO₃, pH 9.6) and coating was allowed to proceed overnight at 4 °C. Plates were washed 5 times in phosphate buffered saline (.137 M NaCl, .003 M KCl, .008 M

Na_2HPO_4 , .001 M NaH_2PO_4) with 0.05% Tween-20 (PBST) prior to addition of fourfold dilutions of mouse sera prepared in PBST with 0.5% gelatin (PBST-g), (Bio-Rad Laboratories Ltd., Mississauga, Ontario). After a 2 hour incubation, plates were washed in PBST and an affinity purified biotinylated goat antimouse Ig (Zymed Laboratories Inc., San Francisco, CA) diluted to 1/5,000 in PBST-g was added to each plate. After an incubation of 60 minutes, plates were washed extensively and Streptavidin-alkaline phosphatase (Gibco, Life Technologies, Burlington, Ontario) diluted to 1/2000, in PBST-g, was added to each plate. Following a 60 minute incubation, plates were washed six times in PBST. Prior to addition of the substrate the plates were washed two additional times in PBS. Development of plates involved the addition of .01 M p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in substrate buffer (0.104 M Diethanolamine-Sigma Chemical Co., 0.5 mM MgCl_2). Absorbances were read on a Model 3550 Microplate Reader (Bio-Rad Laboratories Ltd.) at 30 minutes and again at 60 minutes at 405 nm with a reference wavelength of 490 nm. Antibody isotyping ELISAs were carried out in a similar fashion except that biotinylated goat antimurine IgG_1 , IgG_{2a} , IgG_{2b} , IgG_3 , IgM (Caltag Laboratories, San Francisco, CA) were used at a dilution of 1/8,000 except for IgM (1/2,000). Incubation times with these antibodies was 60 minutes. Addition of Streptavidin-alkaline phosphatase and development of plates was as described for total IgG ELISA.

5.2.6 Spleen and Lymph Node Cell Harvests.

Mouse splenocytes were prepared by mechanically grinding spleens through a

sterile nylon mesh into a sterile Petri dish containing 10-20 mL of RPMI 1640 (Sigma Chemical Co.) This material was then pipetted into sterile 15 or 50 mL tubes and allowed to sit, on ice, for 10 minutes. Debris that settled to the bottom was aspirated with a sterile Pasteur pipette and discarded. Tubes were centrifuged at 250 x g, in a Beckman GPR centrifuge, for 10 minutes (at 4 °C). Red blood cells were lysed by resuspension of the cell pellet in 1.0 mL/spleen of ammonium chloride lysis buffer (0.14 M NH₄Cl and 0.017 M Tris-HCl, pH 7.2) for 60 seconds. Lysis was terminated by addition of 14 mL of RPMI 1640 at room temperature. Samples were pelleted by centrifugation (250 x g) and resuspended in RPMI 1640. Splenocyte numbers were assessed by staining with Trypan blue (Gibco, Life Technologies) and counting on a haemocytometer. Splenocytes were centrifuged (250 x g) and resuspended at a final concentration of 1×10^7 cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co.), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma Chemical Co.), 2 mM L-glutamine (Gibco, Life Technologies,) 1mM sodium pyruvate (Gibco, Life Technologies), 10 mM HEPES (Gibco, Life Technologies) and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.), (complete RPMI-1640). A modification of a method for harvest of Peyers patch lymphocytes was utilized to prepare single cell suspensions from lymph nodes (Spalding, 1983) . Briefly, iliac lymph nodes were collected into 15 mL tubes containing sterile chilled PBSA . Pooled nodes were decanted into a sterile Petri dish and excess PBSA discarded. Nodes were diced with a sterile scalpel in a small volume of CMF solution {0.1 x Hanks Balanced Salt Solution (HBSS), Ca²⁺ Mg²⁺ free; 10 mM Hepes, pH 7.2; 25 mM NaHCO₃,

pH 7.2; and 2% FBS} and then mixed with 10 mL digestion buffer (1 x HBSS, 10% FBS, 15 mM HEPES, pH 7.2) containing 150 Units/mL of Collagenase (CLS 1, Worthington Biochemical Corporation), and 0.015 mg/mL DNase I (Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada). Diced nodes in digestion buffer were transferred to silanized 25 -50 mL Ehrlenmeyer flasks containing a sterile Teflon coated stir bar, and incubated at 37 °C with stirring for 90 minutes. Samples were harvested into sterile 15 mL plastic tubes and allowed to sit for 10 minutes. Large pieces of undigested material were returned to the Erhlenmeyer flask and incubated an additional 30-60 minutes with 5.0 ml of digestion buffer. This material was combined with the initial harvest and cells were centrifuged (250 x g) to pellet. Digestion buffer was decanted and the cells were resuspended in PBSA (GIBCO-BRL). Cells were counted using a haemocytometer and centrifuged (250 x g) one final time. Cells were resuspended to a final concentration of 1×10^7 cells/mL.

A modified protocol for splenocytes was also used that included an additonal 60 minute digestion of pooled splenocytes with collagenase (150 Units/mL of digestion buffer; Sigma Chemical Co.) and DNase I (0.015 mg/ml of digestion buffer; Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada). These digestions were only carried out in experiments that included assessment of antigen-specific responses from cells prepared from lymph nodes. These digestions were carried out prior to cell counts and resuspension.

5.2.7 ELISPOT.

A cytokine specific ELISPOT assay was used as described previously (Czerkinsky et al., 1988; Baca-Estrada et al., 1996). Briefly, single cell suspensions isolated from spleens, lymph nodes or bone marrow were stimulated *in-vitro* by incubating in complete RPMI 1640 at 37 °C and 5% CO₂ for 20 hours in the presence of recombinant authentic gD (0.4 µg/mL). After antigen stimulation, cells were washed twice in complete RPMI 1640 and diluted to a concentration of 1×10^7 cells/mL of complete RPMI-1640. Nitrocellulose plates (FILTApate™, 0.45 µm; Polyfiltronics, Inc., Rockland, MA, USA) were prepared by coating overnight at 4 °C with 2.5 µg/mL of purified antimurine interleukin (IL)-4 (Cat. No. 11B11) or 5 µg/mL of purified antimurine interferon (IFN)-γ (Cat No. R4-6A2) (Pharmingen, San Diego, CA, USA) diluted in carbonate/bicarbonate buffer (45.3 mM NaHCO₃, 18.2 mM Na₂CO₃, pH 9.6). After coating, unbound antibody was washed from the wells thrice with sterile PBST followed by 3 washes with sterile PBS. Wells were blocked for 2 hours at 37 °C with complete RPMI 1640. Blocking media was decanted and 100 µL of antigen stimulated cell suspensions were added to wells in triplicate. After a 20 hour incubation at 37 °C and 5% CO₂, plates were washed 2 times in tapwater and 5 times in PBST to remove cells and nonspecifically bound cytokine. One hundred µL of biotinylated antimurine IL-4 (Cat. No. BVD6-24G2) and IFN-γ (Cat. No. XMG1.2) (Pharmingen Canada Inc.) monoclonal antibodies (3 µg/mL of each in 1% FBS/PBS) were added to appropriate wells and incubated for 4-6 hours at ambient temperature on an oscillating mixer. Plates were washed 6 times in PBST and

100 μ L of a 1/1000 dilution of Streptavidin alkaline phosphatase (Bio/Can Scientific, Mississauga, Ontario, Canada) in 1% FBS/PBS was added to each well. After a 2 hour incubation, at ambient temperature, the plates were washed as before with 2 final washes in PBS. Plates were developed by the addition of 50 μ L of the substrates, 5-bromo-4-3-indolyl phosphate (BCIP), and nitroblue tetrazolium (NBT), (Moss Inc., Pasadena, MD). Plates were allowed to develop at ambient temperature for 10-30 minutes, after which reactions were stopped by extensive washing in distilled water. After air drying, spots were counted under a dissecting microscope.

B-cell ELISPOT involved the use of nitrocellulose plates (Millipore Milliscreen-HA, Millipore, Bedford, MA) that were coated overnight at 4 °C with purified recombinant truncated gD at 5.0 μ g/mL in carbonate/bicarbonate buffer (pH 9.6). Coated plates were washed three times in sterile PBST followed by 3 washes in sterile PBS. Nonspecific binding sites were then blocked with complete RPMI 1640 at 37 °C for 1-2 hours. Fresh, unstimulated cells from spleen or lymph nodes, prepared as described above, were added in a volume of 100 μ L (1×10^7 cells/mL) to each well. To account for nonspecific binding of IgG uncoated, but blocked, control wells also received cells from each test sample in triplicate. Plates were incubated at 37 °C with 5% CO₂ for 10-12 hours. After the incubation period, all cells were washed out of the wells by 2 initial tapwater washes followed by 6 PBST washes. Biotinylated antimurine IgG₁ or IgG_{2a} antibodies (1/8,000 dilution) were added in a 100 μ L volume of PBST (with 1% FBS) and incubated with mixing for 3-4 hours at ambient temperature. After washing in PBST six times,

streptavidin-alkaline phosphatase (1/1,000 dilution in PBS-T with 1% FBS) was added to each well (100 μ L). After a 60 minute incubation, plates were washed six times in PBST and 2 times in PBS. Plates were developed as described for the cytokine ELISPOT.

5.2.8 Statistical Analysis.

Data was entered into a database in the statistical analysis program InStat GraphPad[™]. Differences in antibody titers among vaccine groups were investigated using the nonparametric Mann-Whitney *U* test.

5.3 Results

5.3.1 Enhanced development of humoral immunity following DNA immunisation.

In a preliminary experiment we established that some passively immune C3H/HeN mice immunized with a plasmid encoding a secreted form of BHV-1 gD were capable of mounting significant levels of humoral immunity (Lewis et al., 1997). We expanded this study and also assessed if co-administration of plasmid encoded murine GM CSF would enhance humoral responses in passively immune mice following DNA immunization. Figure 5.1 D depicts extremely rapid development of active antibody titers in most of the immunized mice, despite (perhaps even because of) the presence of pre-existing passive polyclonal antisera at the time of immunization. Four week IgG titers clearly show 80% of immunized mice seroconverted with a 90% seroconversion rate at 6 weeks.

Co-administration of a 25 µg dose of plasmid encoded murine GM-CSF (pSLRSV.GM-CSF) with pSLRSV.SgD in female C3H/HeN mice resulted in some enhancement of seroconversion efficiency and magnitude of serum antibody responses (Fig. 5.1 C vs E). Only one mouse failed to seroconvert in the pSLRSV.SgD plus pSLRSV.GM-CSF vaccine group at 2 weeks post-immunization as compared to three nonresponders in mice receiving pSLRSV.SgD only (Fig. 5.1 E and C, respectively). Serum antibody levels were also significantly greater ($p \leq 0.05$) in the pSLRSV.SgD plus pSLRSV.GM-CSF vaccine group as compared to the pSLRSV.SgD group at 4 weeks. A 2 fold augmentation of splenic IFN γ and a 7 fold increase in IL-4 secretion levels following immunization with pSLRSV.SgD plus pSLRSV.GM-CSF was also evident (Fig. 5.2). This data is consistent with earlier experiments which assessed a dose range of plasmid encoded GM-CSF on immune responses in mice following co-administration with an optimized dose of pSLRSV.SgD (unpublished data). Interestingly, co-administration of this dose of plasmid encoded GM-CSF with pSLRSV.SgD did not enhance seroconversion efficiency or the magnitude of serum IgG levels in passively immune mice (Fig. 5.1 F). Indeed, inclusion of pSLRSV.GM-CSF appeared to delay the onset of active humoral responses and diminished the seroconversion efficiency relative to mice immunized with pSLRSV.SgD alone. The suppressive effect of co-administered pSLRSV.GM-CSF, in the context of pre-existing passive anti-gD serum titers, is consistent with cytokine ELISPOT data presented in Figure 5.2. While the splenic cytokine profile was predominantly IL-4, in the presence or absence of passive titers, the magnitude of both IL-4 and IFN γ in mice receiving pSLRSV.SgD/pSLRSV.GM-CSF

Fig. 5.1 Effect of passive immunity on induction of active responses by DNA immunization. C3H/HeN mice immunized with pSLRSV.SgD in the face of passively administered hyperimmune sera develop active humoral responses within 2 weeks of intramuscular immunization. Hyperimmune sera for passive transfer to recipient mice (B, D, F and G) was prepared as described in Materials and Methods. Hyperimmune serum was injected into the intraperitoneal cavity on Day -2. Recipient serum IgG titers were read by ELISA on Day -1. Vaccines VSA3/rtgD (**A, B**), pSLRSV.SgD (**C and D**) and pSLRSV.SgD plus pSLRSV.GM-CSF (**E and F**) were administered at Day 0 intramuscularly or subcutaneously (VSA3/rtgD). Doses of vaccines were: 200 ng rtgD (**A and B**), 50 µg/quadriceps muscle pSLRSV.SgD (**C,D,E and F**) and 12.5 µg/quadriceps pSLRSV.GM-CSF (**E and F**) mixed and co-administered with the pSLKRSV.SgD. All experimental groups contained 10 mice except for groups receiving VSA3/rtgD (five mice/group). Graph **G** represents geometric mean and standard error of the mean for 10 mice receiving hyperimmune sera and no vaccination. Data is represented for individual animals. ELISA values are depicted as absorbances (405 nm) at a 1/200 serum dilution. Individual mice from vaccine + passive antibody groups were considered seropositive if the optical density (O.D. _{405 nm}) scored a minimum of one standard deviation or better above the corresponding value in Graph **G**.

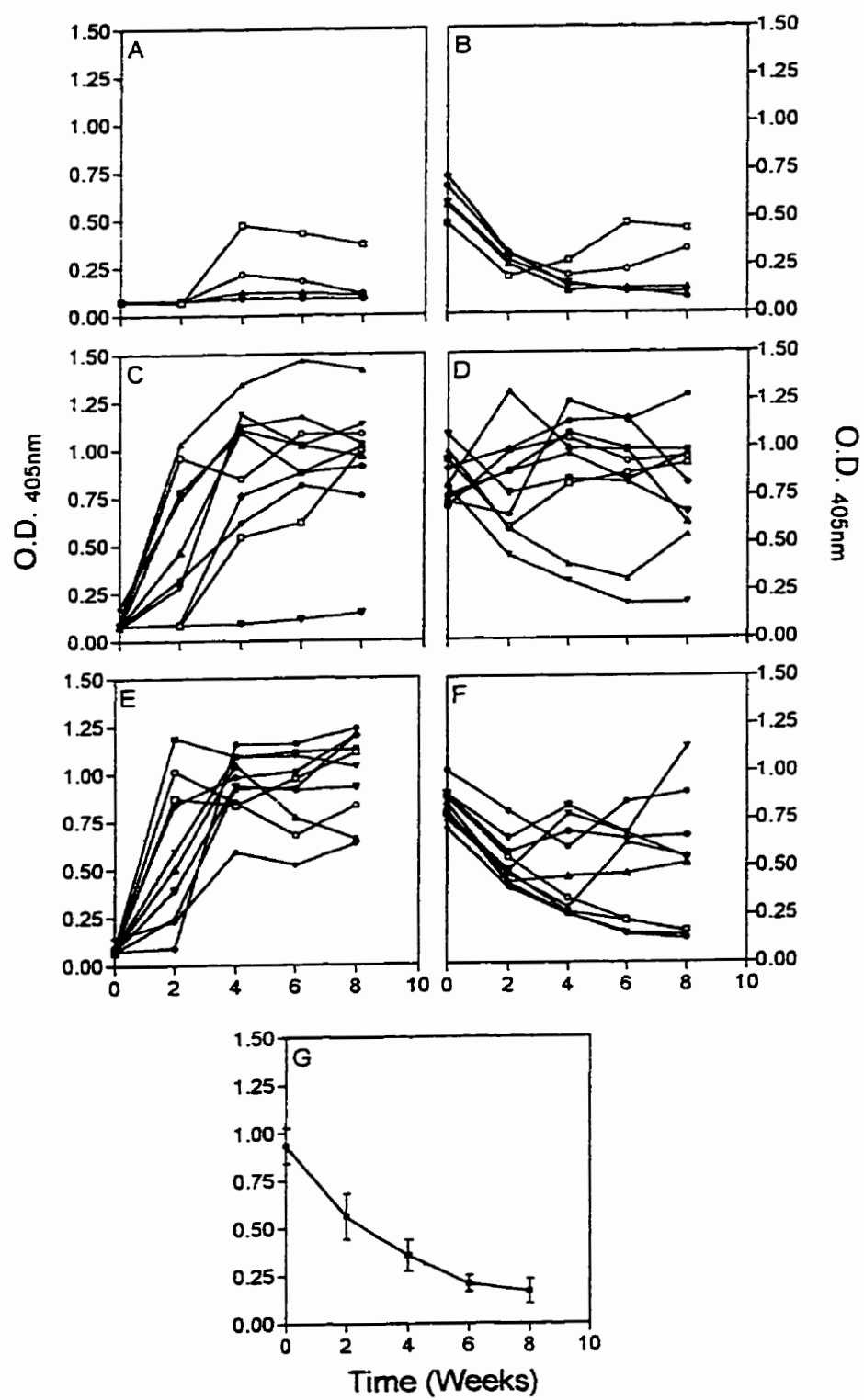
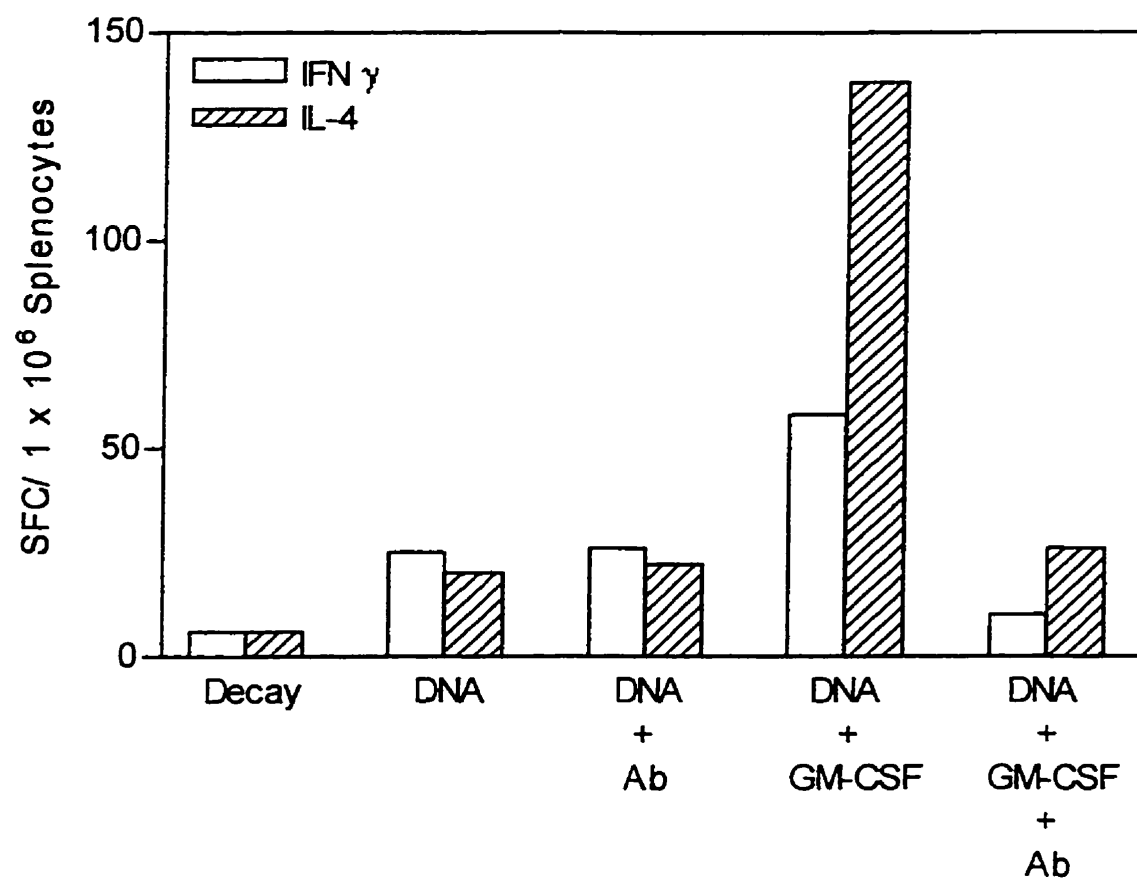


Fig. 5.2 Splenic cytokine profile in passively immune mice immunized with a DNA-based vaccine. Splenic cytokine profiles in mice immunized with pSLRSV.SgD is similar regardless of the presence of passively administered hyperimmune sera. The mean number of cytokine-secreting cells following *in vitro* restimulation of splenocytes with authentic, full-length, gD is depicted. Splenocytes from mice illustrated in Fig. 5.1 including: controls (Hyperimmune serum only) or DNA immunized mice, were harvested 9 weeks after a single intramuscular immunization. Data groups are depicted as: **Decay** (Control groups receiving hyperimmune serum only), **DNA** (pSLRSV.SgD), **DNA + Ab** (Hyperimmune sera followed by pSLRSV.SgD), **DNA + GM-CSF** (pSLRSV.SgD/pSLRSV.GM-CSF) and **DNA + GM-CSF + Ab** (Hyperimmune sera followed by pSLRSV.SgD/pSLRSV.GM-CSF). Mean values for each group were determined from triplicate wells of pooled splenocytes from seropositive, or control, mice.



was approximately 4 fold greater than mice immunized with these constructs in the face of pre-existing serum titers. Mice immunized once with a suboptimal dose of the conventional vaccine formulation VSA3/rtgD showed little difference in seroconversion efficiency or serum IgG levels in the presence or absence of pre-existing titers (Fig. 5.1 A and B).

5.3.2 Effect of MAb on the development of active humoral immunity following DNA immunization.

Since antibody isotype may influences the development of immune responses groups of mice were passively immunized with hyperimmune antisera or anti-gD monoclonal antibodies of different (IgG₁ or IgG_{2a}) isotypes. The most striking data obtained was clear and significant ($p \leq 0.05$) evidence that the isotype of the passive antibody has a direct impact on the induction of active humoral immunity following immunization with a DNA-based vaccine (Fig. 5.3 and 5.4). Figures 5.3 E and G illustrate that the pretreatment of C3H/HeN mice with MAb PB136 (IgG₁) suppresses the efficacy of active humoral immunity while pretreatment with MAb 1G6 (IgG_{2a}) augmented the magnitude and seroconversion efficacy of humoral immunity (Fig. 5.3 F). Two weeks after DNA immunization serum IgG ELISA data show that 6 of 8 mice developed active titers when the pre-existing passively administered anti-gD antibody was a monoclonal of the IgG_{2a} isotype (1G6) (Fig. 5.3 F). Conversely, only 1 of 8 mice immunized with pSLRSV.SgD in the face of pre-existing titers of PB 136 (IgG₁) seroconverted during the same time period (Fig. 5.3 E). A mixture of 3 IgG₁ MAbs

Fig. 5.3 Effect of monoclonal antibodies on active humoral immunity after DNA immunization. Development of active humoral responses, following immunization of female C3H/HeN mice with pSLRSV.SgD, is affected by the isotype of pre-existing passive titers of monoclonal antibodies. Hyperimmune sera was injected intraperitoneally on Day -2 at Low (Ab_{Lo}) or High (Ab_{Hi}) doses. Monoclonal antibodies PB136, 3D9, 2C8 (IgG_1) and 1G6 (IgG_{2a}) were diluted into normal saline prior to intraperitoneal injection on Day -2. Mice were immunized intramuscularly with pSLRSV.SgD at 50 μ g/quadriceps once on Day 0. Vaccination with DNA was carried out in the absence of passive titers (**B**) or in the presence of Low (**C**) or High (**D**) doses of hyperimmune sera. Mice were also immunized with pSLRSV.SgD in the presence of passive titers of the Mabs: PB136 (**E**), 1G6 (**F**) or a combination of PB136, 3D9 and 2C8 (**G**). Control groups (**A**) represent geometric means and standard deviations for mice receiving High or Low doses of hyperimmune sera or Mabs representing IgG_1 (PB136) or IgG_{2a} (1G6) isotypes. Data are represented as absorbances at 405 nm for individual mice (except in Control groups), at serum dilutions of 1/400. Individual mice from vaccine + passive antibody groups were considered seropositive if the optical density (O.D. $_{405\text{ nm}}$) scored a minimum of one standard deviation or better above the corresponding value in Graph A.

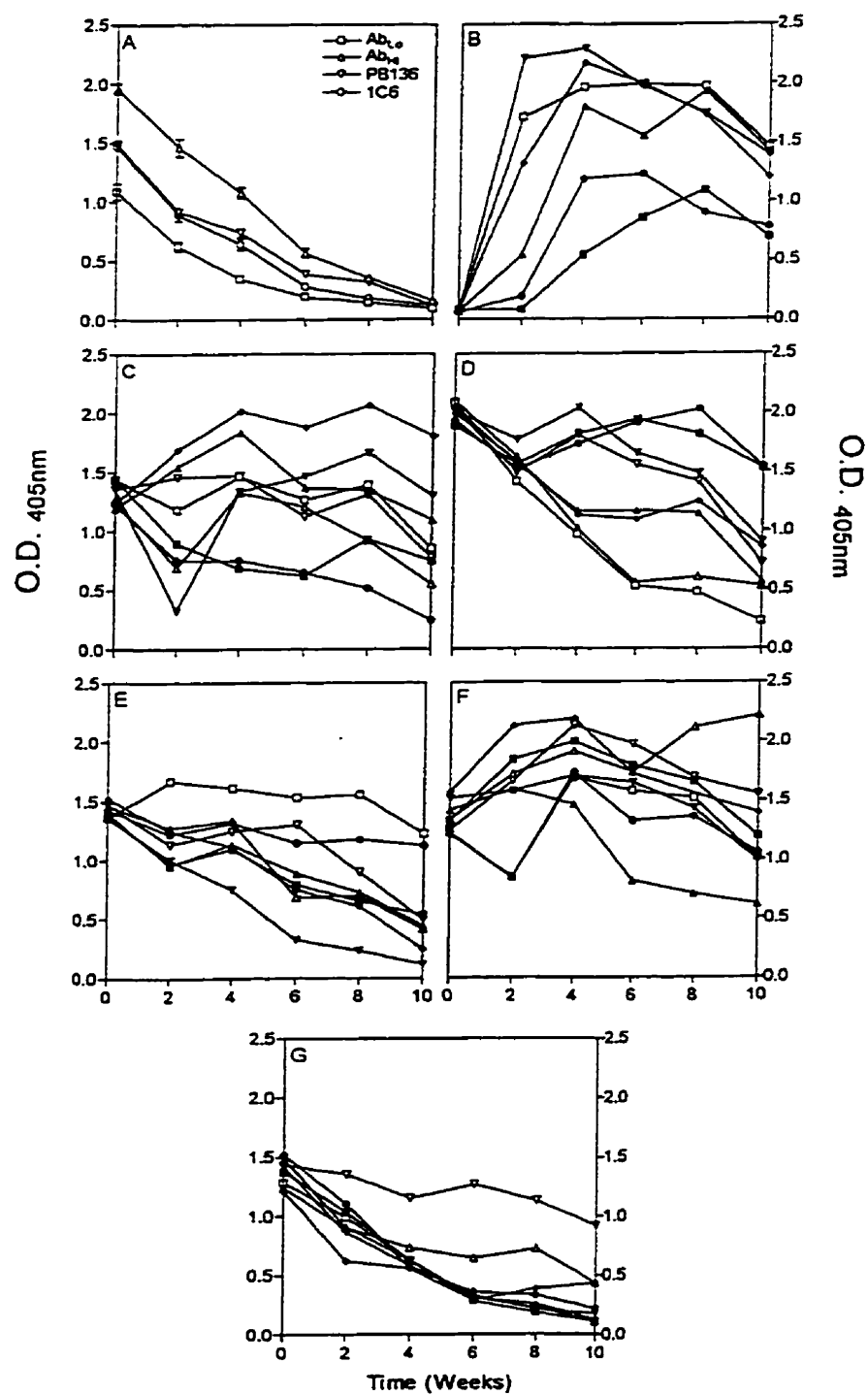
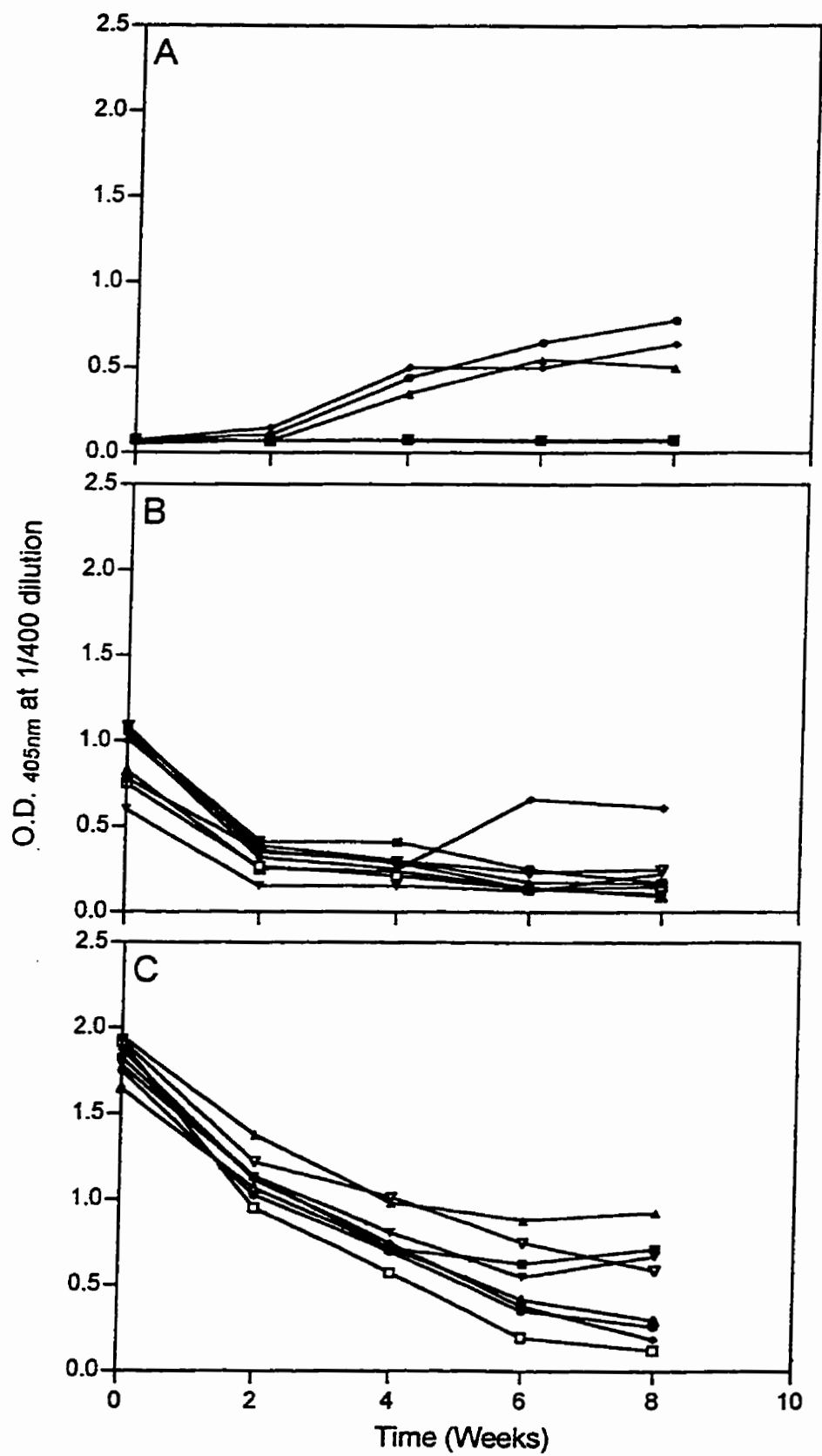


Fig. 5.4 Active humoral immunity develops in passively immune mice following immunization with a conventional vaccine. **Graphs A** (VSA3/rtgD), **B** (VSA3/rtgD + Ab_{Lo}) and **C** (VSA3/rtgD + Ab_{Hi}) depict conventional vaccine controls for the experiment described in Fig. 5. A single dose of 1.0 µg/mouse, in VSA3, was administered subcutaneously to all mice at Day 0. Data is represented as absorbances at 405 nm for individual mice read at serum dilutions of 1/400. Groups depicted in **B** and **C** include data from each of 8 mice, while the VSA3/rtgD group (**A**) included only 5 animals.



shown to recognize different epitopes on BHV-1 gD, showed only 2 of 8 mice seroconverting during the first 8 weeks of the experimental period with only one maintaining measurable active titers at 10 weeks (Fig. 5.3 G).

Immunization of mice with pSLRSV.SgD in the presence of pre-existing High (Fig. 5.3 D) and Low (Fig. 5.3 C) polyclonal antibody titers showed similarities in seroconversion efficacy and magnitude of serum antibody response. The results observed with mice immunized in the face of passive polyclonal hyperimmune sera were similar to those observed in a separate experiment depicted in Figure 5.1 D. Mean titers for both High and Low passive antibody titer groups were statistically ($p \leq 0.05$) lower than mean titers observed for mice immunized in the face of pre-existing passive antibody titers of MAb 1G6 (isotype IgG_{2a}). Seroconversion efficacy appeared similar between Mab 1G6 (IgG_{2a}), Low dose polyclonal and High dose polyclonal antibody at 4, 6 and 8 weeks post-immunization.

Figure 5.4 summarizes data for mice immunized with VSA3/rtgD (1.0 µg/mouse) in the absence (Fig. 5.4 A) and presence of high (Fig. 5.4 C) and low (Fig. 5.4 B) doses of pre-existing titers of polyclonal antisera. While this relatively high conventional vaccine dose is still suboptimal (in the absence of boosting) (Baca-Estrada et al., 1996), there is a suggestion that some enhancement of immune response occurred within the high dose hyperimmune antibody group and is indicated by a 50% seroconversion rate depicted in Figure 5.4 C (VSA3/rtgD + Ab_H) as compared to Figure 5.4 B (VSA3/rtgD + Ab_L).

5.3.3 Mixed Th1/Th2 immunity following DNA immunization of passively immune C57BL/6 mice.

C3H/HeN and C57BL/6 mice immunized with pSLRSV.SgD show a predominance of IgG₁ in sera (see section 3.0). However, C3H/HeN mice respond inconsistently to truncated gD formulated in VSA3 (see sections 3.3.2 and 4.3.1). Immune responses to VSA3:gD have been described in C57BL/6 mice (Baca-Estrada et al., 1996). To ensure that a proper conventional vaccine control model was utilized we decided to assess the issue of passive antibody impact on the induced immune response in C57BL/6 mice. In this experiment we utilized a suboptimal dose of 0.1 ug/mouse of truncated gD formulated with the adjuvant VSA3. This dose has been demonstrated to elicit poor B-cell responses, even following a boost, but could prime splenic and lymph node T-cell responses following subcutaneous immunization. To our surprise this dose of VSA3:rtgD, in the face of pre-existing levels of passively administered hyperimmune sera resulted in 4 of 8 immunized animals developing moderate to high serum antibody levels (Fig. 5.5 E). This data strongly suggests that that the hyperimmune serum utilized to passively immunize 6-7 week old C57BL/6 mice enhanced, rather than suppressed, the development of humoral immunity to the conventional vaccine. Figure 5.6 A demonstrates that splenic recall IFN γ and IL-4 responses did not differ greatly in the presence and absence of pre-existing passive titers of hyperimmune anti-gD antibodies. Figure 5.6 B represents cytokine profiles in the iliac nodes and shows significant levels of recall IL-4 with little of no IFN γ in the pool samples from mice immunized with VSA3:rtgD in the presence of passive hyperimmune antisera. This was somewhat

Fig. 5.5 Impact of mouse strain on the development of active immunity in passively immune mice following DNA immunization. Development of humoral immune responses in the face of passive titers of hyperimmune sera appears to be enhanced following immunization with pSLRSV.SgD or a conventional vaccine. C57BL/6 mice were immunized with pSLRSV.SgD (50 µg/quadricep) (**B + C**) or VSA3/rtgD (100 ng/mouse) (**D + E**) in the presence or absence of passive titers of gD antisera. A single dose of hyperimmune antisera was administered to mice (**A, C and E**) by intraperitoneal injection on Day -2. Recipient mouse serum titers were measured by ELISA on Day -1. Vaccines were administered on Day 0, with no subsequent boosts. Serum samples were collected at 2, 4 and 6 weeks post immunization and total serum IgG levels were determined by ELISA. Data are depicted for 8 or 10 individual mice within each group and represented by absorbances (405 nm) at serum dilutions of 1/160. Individual mice from vaccine + passive antibody groups were considered seropositive if the optical density (O.D. _{405 nm}) scored a minimum of one standard deviation or better above the corresponding value in Graph A.

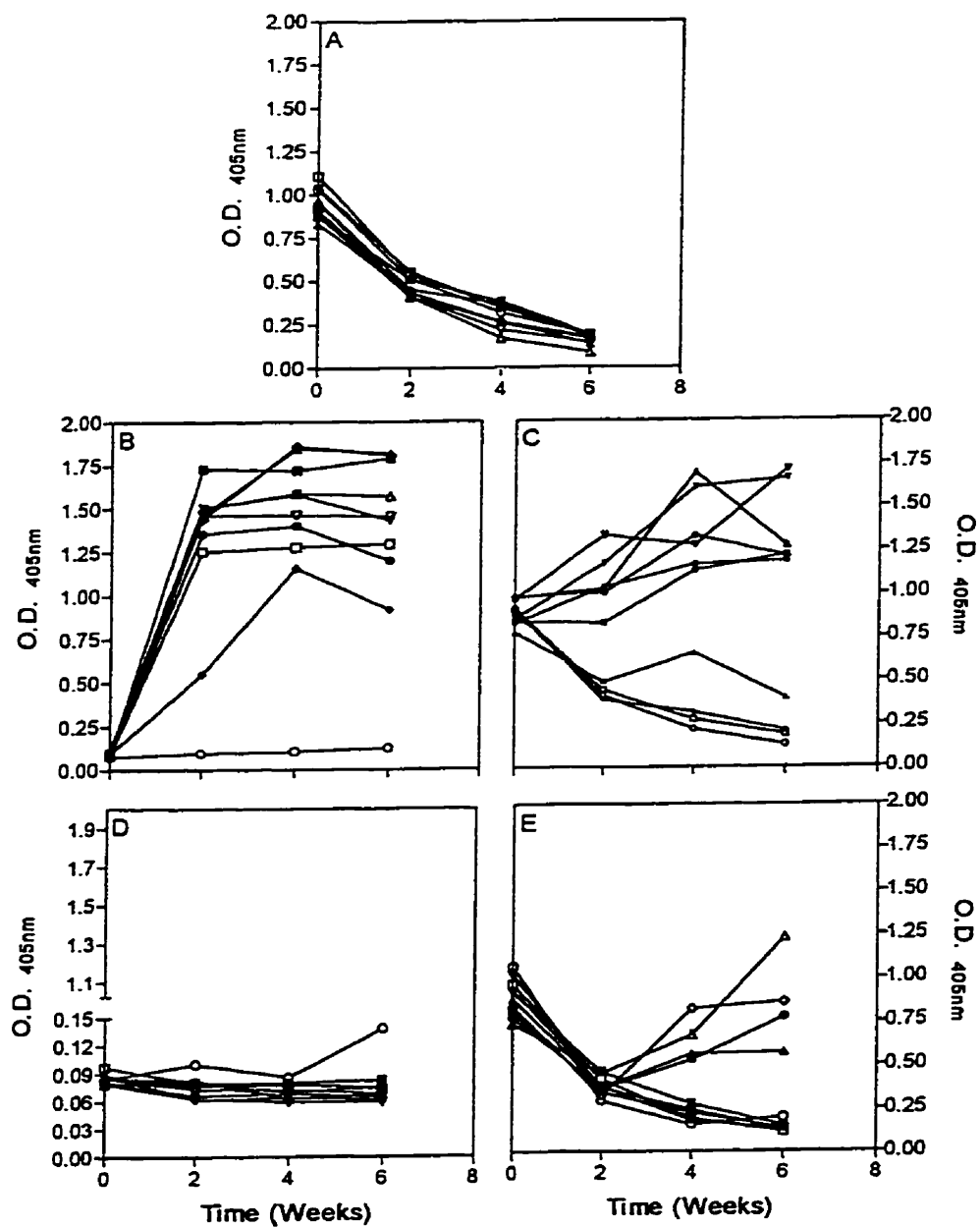
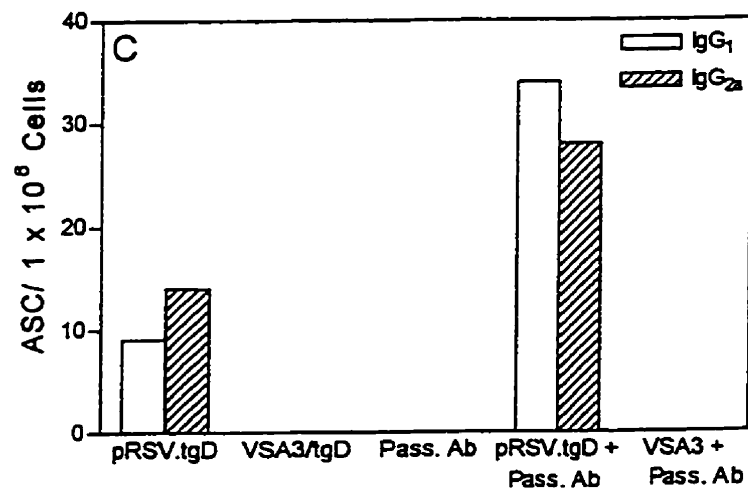
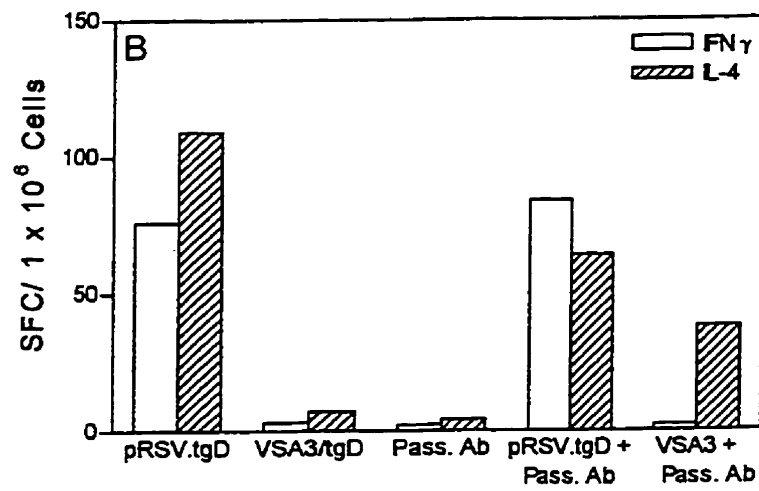
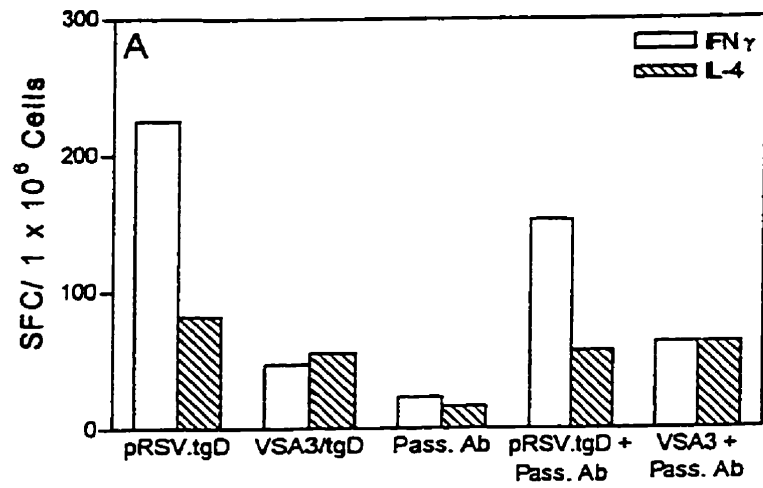


Fig. 5.6 Effect of passive immunity on cytokine and antibody secretion profiles following DNA immunization. Splenic and iliac lymph node cytokine and antibody isotype profiles are similar regardless of the passive titer status at the time of immunization with pSLRSV.SgD. Pooled cells were prepared from spleens and retroperitoneal iliac lymph nodes harvested at 7 weeks post immunization from seropositive C57BL/6 mice. Figures **A** (Spleen) and **B** (Iliac node) depict IL-4 and IFN γ secreting cells/ 10^6 cells following 20 hours restimulation, *in vitro*, with authentic full length gD. Cytokine and B-cell isotype ELISPOT (**C**) results are expressed as the mean of triplicate wells from pooled cells from 10 (Control passive hyperimmune-Decay-group), 9 (pSLRSV.SgD), 7 (pSLRSV.SgD + Ab), 5 (VSA3/rtgD) and 4 (VSA3/rtgD + Ab) mice from each group.

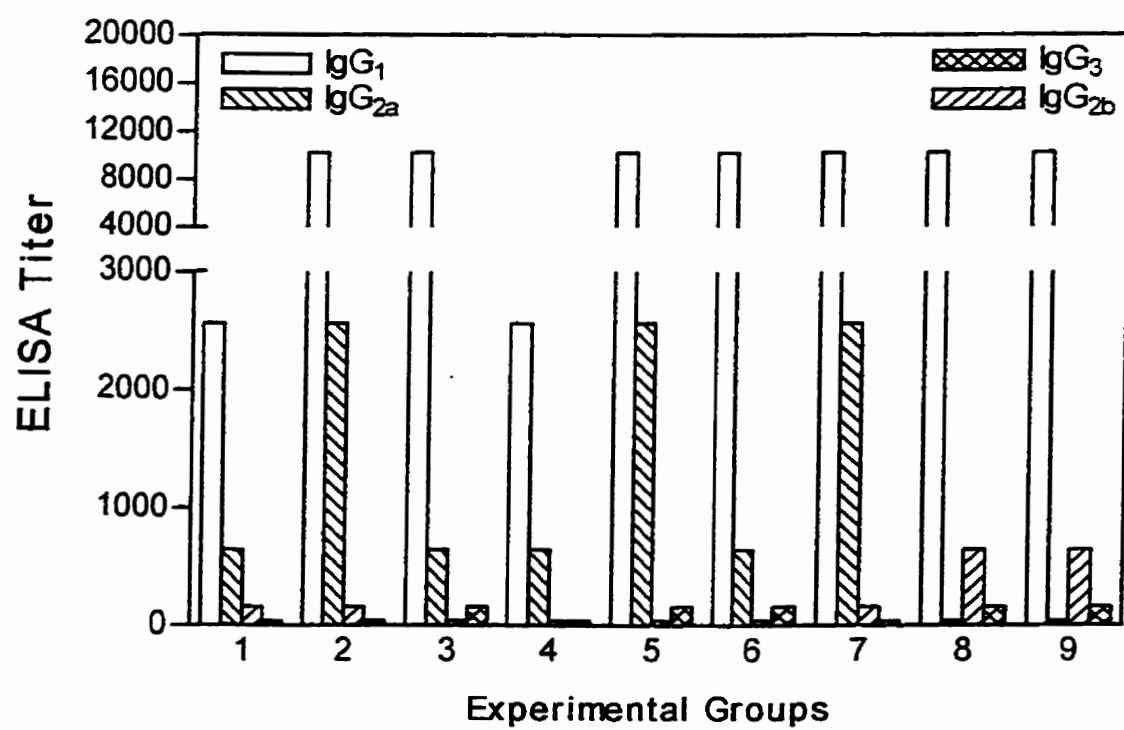


surprising because the iliac lymph node does not drain the subcutaneous immunization site utilized for the VSA3rtgD vaccine as indicated by the relative absence of IL-4 or IFN γ secretion from lymph node cell pool collected from untreated mice immunized with VSA3/rtgD (Fig. 5.6 B). This information suggests that pre-existing passively administered antiserum was responsible for a fundamental difference in antigen distribution following immunization. However, it is unknown why T-cell responses would have occurred within this nondraining lymph node compartment and not B-cell responses (Fig. 5.6 C).

Not surprisingly, C57BL/6 mice immunized one time with 100 μ g pSLRSV.SgD, in the presence or absence of passive serum titers to BHV-1 gD, generated humoral immune response of greater magnitude and consistency than mice immunized with the suboptimal dose of VSA3/rtgD (Fig. 5.5 B and C). Splenic cytokine profiles were consistent with previous experiments in that recall levels of IFN γ exceeded those of IL-4 (Figure 5.6 A). Draining lymph node cytokine profiles showed a ratio of IFN γ :IL-4 that approached 1:1 and was distinct from the splenic cytokine profile in this regard. This diminished level of IFN γ relative to iliac node IL-4 levels, was likely responsible, in large part, for the predominance in serum IgG₁ in mice immunized with plasmid encoding the truncated version of BHV-1 gD (Figure 5.7).

Figure 5.7 summarizes the serum antibody isotype in seropositive mice following immunization with pSLRSV.SgD in the absence and presence of passively administered antisera. This figure illustrates that the mouse strain had a greater impact on serum IgG isotype profile than pre-existing passive titers (C57BL/6 mice in Groups 8 and 9)

Fig. 5.7 Influence of mouse strain on serum isotype profiles. Serum isotype ELISA profiles are affected by mouse strain and not the presence of passive titers of polyclonal or monoclonal antisera. Pooled seropositive sera from mice depicted separate experiments depicted in Figures 5.3 (Groups 1 and 2), 5.5 (Groups 3-7) and 5.7 (Groups 8 and 9) were assessed for serum IgG isotype by isotyping ELISA. Groups 1 and 2 represent female C3H/HeN mice immunized with pSLRSV.SgD and pSLRSV.SgD + Passive hyperimmune sera, respectively. Groups 3-7 represent data from female C3H/HeN mice immunized with pSLRSV.SgD, pSLRSV.SgD + Low dose hyperimmune sera, pSLRSV.SgD + High dose hyperimmune sera, pSLRSV.SgD + Mab PB136 (IgG₁), and pSLRSV.SgD + Mab 1G6 (IgG_{2a}), respectively. Groups 8 and 9 represent serum ELISA isotype data from female C57BL/6 mice immunized with pSLRSV.SgD and pSLRSV.SgD + hyperimmune sera. All serum samples were collected from mice at 6 weeks postimmunization. Data is represented by absorbances (405 nm) at a serum dilutions of 1/160.



However, serum IgG₁ consistently predominated in all vaccine groups with some suggestion that IgG_{2a} levels were elevated in mice treated with high doses of hyperimmune sera or MAb 1G6 (IgG_{2a}).

5.4 Discussion

In this study we evaluated the ability of pSLRSV.SgD, a DNA-based vaccine encoding a secreted version of BHV-1 gD, to elicit active immunity in passively immune C3H/HeN or C57BL/6 mice. Potent active humoral immunity was evident within 2–4 weeks of immunization regardless of the passive immune status of injected mice. Indeed, in this particular model, there was clear evidence that the presence of passive antibodies appeared to augment the development of the active immune response to pSLRSV.SgD, as well as, a subunit vaccine formulation.

At least two recent articles describe use of DNA-based vaccines to circumvent maternal antibody suppression (Monteil et al., 1996; Hassett et al., 1997). Monteil et. al. (1996) utilized day old piglets from immune and nonimmune dams and demonstrated that following i.m. injection with 400 ug of plasmid encoding pseudorabies virus glycoprotein D, piglets from immune dams did not develop serum antibodies or demonstrate any evidence of priming. Furthermore piglets from nonimmune dams developed moderate serum antibody titers only following a boost at 6 weeks postpartum.

Hassett et. al. (1997) immunized murine neonates from immune and nonimmune dams i.m. with 50 µg of plasmids encoding the nucleoprotein (NP) from LCMV

(Lymphochoriomeningitis virus). These authors showed that, following sublethal challenge, approximately 50% of neonates from immune and nonimmune dams developed MHC-restricted, Np-specific CTLs more rapidly than unprimed animals. Further, approximately 95% of these immune neonates showed reductions in splenic viral titers following challenge. These authors argued that maternal antibodies did not interfere with the development of CTL responses possibly due to the fact that LCMV-NP is an intracellular protein that would not come into direct contact with serum antibody. These authors did not determine if humoral immunity was induced in this model because LCMV-specific CTL have been demonstrated to be adequate and sufficient to confer protection against viral challenge. These studies are compelling because they demonstrated that compartmentalization of the antigen may serve to circumvent some of the problems associated with maternal antibody suppression of immunity. However, this approach would be restricted to pathogens that can be reliably and completely cleared with CTL responses only (Zinkernagel, 1996). Unfortunately these authors did not include a conventional vaccine control, such as modified live LCMV or NP formulated with adjuvants such as ISCOMs or liposomes known to facilitate potent CTL responses (Cox and Coulter, 1997). In light of the suboptimal efficacy described for mice following immunization with plasmid encoded LCMV-NP this comparison is critical before any claims of efficacy of DNA-based vaccines can be made. Several other publications describe the impact of DNA-based vaccines on neonatal immune responses (Bot et al., 1996; Mor et al., 1996; Wang et al., 1997). While two of these publications describe the successful development of T-cell responses in neonatal mice neither addressed the

influence of maternal antibody on the development of active humoral immunity or the mechanism whereby passive antibody suppresses active B-cell responses to antigen.

The mechanism for *in vivo* B-cell failure to respond to Ag in the presence of antigen-specific maternal or passive antibody in neonates is unknown. However, recent evidence suggests that simultaneous signalling through the B-cell receptor (BCR) and the FcγRIIB1 receptor on naive B-cells results in a negative signal delivered to the potential antigen-specific B-cells that prevents not only secretion of antigen-specific IgM but also inhibits the maturation of these B-cells into IgG secreting plasma cells and presumably inhibits the development of a memory B-cell population (Phillips and Parker, 1983). Several recent articles demonstrate that co-ligation of these two receptors on naive/pre B-cells results in phosphorylation of Tyrosine³⁰⁹ of the 13 a.a. immunoreceptor tyrosine-based inhibition motif (ITIM) found within the cytoplasmic domain of receptor FcγRIIB1 (Muta et al., 1994; Ravetch, 1997). This phosphorylation event initiates an inhibitory cascade that commences with recruitment of one of several candidate phosphatases (SHIP and/or PTP1C) that modulate inhibition of cellular calcium influx or mobilization of intracellular calcium stores required for BCR mediated signalling (D'Ambrosio et al., 1995; Ono et al., 1996). B-cells in mice express only the low affinity FcγRIIB1. Interestingly, FcγRIIB1 receptor knockout mice show hyperimmunoglobulinemia in response to both T-cell dependent and T-cell independent antigens (Takai et al., 1996). Thus, it appears that B-cell FcγRIIB1 coligation with BCR may mediate a feedback inhibition which prevents excessive expansion of B-cell responses to a given antigen as long as serum IgG antibody levels remain high (Tew et

al., 1997).

Our initial premise was that expression of plasmid encoded antigen from *in vivo* transfected cells would persist beyond the point where serum antibodies, in passively immune mice, waned to noninhibitory levels. In addition, DNA-based vaccines may induce the development of active humoral responses prior to waning passive titers and increase the likelihood of eliciting potent cell-mediated immunity. Recent evidence suggests that the efficacy of DNA-based vaccines may stem from the proximity of antigen presenting cells (bearing injected plasmid sequences encoding antigen) and/or antigen expression to naive T and B-cell populations (Steinman et al., 1997; Tew et al., 1997). This proximity is suggested by several recent articles implying that local and possibly lymph node resident dendritic cells may be of pivotal importance in the efficient development of ensuing immune responses (Pertner et al., 1995; Condon et al., 1996; Manickan et al., 1997; Roman et al., 1997). If this is indeed the case, then trafficking to draining lymph nodes would allow direct presentation of antigen to naive T and B-cells without significant exposure to pre-existing antibody.

The rapid development of active humoral immunity described by our data suggests that longevity of antigen expression following DNA immunization is not an issue in this model. Figures 5.1 D, 5.3 C and D, and 5.5 C clearly demonstrate that C3H/HeN mice immunized with plasmid pSLRSV.SgD develop active humoral immunity regardless of the presence of high or low doses of passive hyperimmune sera. There are a number possible explanations for this occurrence other than the discussed advantages of *in vivo* transfected dendritic cells and proximity to naive T and B-cells within the draining

lymphoid tissues. For example, it has been demonstrated that passive titers of IgM do not suppress the development of active B-cell responses, indeed, this isotype has been demonstrated to enhance the developing humoral immune responses relative to vaccine controls (Henry and Jerne, 1968). A more comprehensive, albeit evolving, understanding of the inhibitory signalling occurring in B-cells allows one to hypothesize that IgM:Ag complexes prevent inhibitory B-cell signalling by interfering with the ability to co-ligate FcγRIIB1 and BCR receptors. It has been demonstrated that syngeneic idiotype presentation in the context of IgM is highly immunogenic while presentation of the same idiotype in the context of Ig isotypes, IgG₁, IgG_{2a} and IgG₃ leads to antigen-specific tolerance (Reitan and Hannestad, 1995). Indeed it appears likely that complement fixation by the polymeric IgM is directly involved in the ensuing humoral response (Heyman et al., 1988). Indeed mutation of serine⁴⁰⁶ within the third constant domain of IgM ablates the complement fixation capacity and corresponds to a significant reduction in the ability of IgM:Ag complexes to facilitate thymus-dependent humoral immune responses (Shulman et al., 1986; Heyman et al., 1988). The specific mechanism by which IgM drives the development of T-dependent B-cell responses appears to involve direction of the Ag:IgM:C3d (iC3d) complexes to CD21 receptors on FDC and B-cells (up to the centrocyte stage) for subsequent presentation to primed CD4⁺ T-cells. CD21 and C3 knockout mice, as well as a complement deficient breed of dogs, have been demonstrated to be defective in the ability to mount normal humoral immune responses to infection or vaccination with T-dependent antigens (O'Neil et al., 1988; Carroll and Fischer, 1997). Furthermore, pretreatment of mice with MAbs directed against CR2 or a soluble

CR2:IgG1 chimeric molecule results in suppression of antibody responses to a T-dependent antigen (Hebell et al., 1991). Indeed it has been demonstrated recently that C3d polymer fusions to hen egg white lysozyme enhanced serum antibody responses by 100 to 10000 fold (Dempsey et al., 1996). Thus it appears that substantial evidence exists that describes a fundamental role of complement component C3 in the induction of T-dependent B-cell responses.

Despite the evidence for the potential roles of IgM and complement in circumvention of passive antibody mediated B-cell suppression we feel that contribution of passive IgM to the development of active humoral immunity is minimal. For example, although there were low titers of IgM in donor sera utilized in these experiments, a 3-5 hour serum half-life for this isotype would effectively ensure only minute amounts of antigen-specific passive IgM would be present at the time of immunization (Vieira and Rajewsky, 1988). However, Figure 5.3 F suggests that antibody isotype IgG_{2a} may provide some potential for facilitating circumvention of suppressive pre-existing passive titers. Certainly there is evidence suggesting that IgG_{2a} possesses a much greater capacity to fix complement than IgG₁ (Klaus et al., 1979; Ishizaka et al., 1995). However, there is *in vivo* data, based on an idiotype switch variant model, which demonstrates that IgG_{2a}, IgG₁, and IgG_{2b} failed to enhance the immunogenicity of the presented idiotype (Reitan and Hannestad, 1995). This work suggests it may not be the complement fixation capacity of IgG_{2a} that is responsible for the augmented humoral immunity observed in Figure 5.3 F.

It has also been suggested that affinity differences in passive antibodies may have a

significant impact on the immune outcome following immunization (Keller et al., 1991).

While a plausible mechanism for how differences in passive antibody affinity can determine outcome to immunization is unknown, it has been documented that high affinity monoclonal antibodies tend to suppress the development of humoral immunity while lower affinity monoclonals have little or no effect (Keller et al., 1991). However, it should also be noted that this information was acquired using only monoclonal antibodies of the IgG₁ isotype and showed at least one high affinity monoclonal that failed to suppress the development of humoral immunity. Affinity differences between IgG₁ and IgG_{2a} antibodies described in Figures 5.3 E,F and G may also play a role in modulating the immune response.

Ultimately the apparent inability of low affinity MAb to suppress B-cell responses may involve a natural antibody or BCR facilitated displacement of antigen bound, low affinity MAb. This displacement would effectively eliminate the FcγRIIB1-BCR coligation signal necessary for inhibitory B-cell activation. Regardless, the polyclonality of maternally derived antibodies, in the context of maternal immunization regimes of pathogen exposure, make affinity contribution to inhibition or enhancement of responses to neonatal vaccination difficult to explain. A more plausible explanation, although relatively unexplored, involves described Fc affinity differences for the high affinity FcγRI (CD 64) receptor present on blood borne professional antigen presenting cells (Tew et al., 1997). Fc receptor distribution is well documented, and there is some evidence that targeting to the high affinity receptors on nonphagocytic (maturing) dendritic-like cells by monomeric Ab:Ag complexes can circumvent the "normal" B-cell

downregulation upon exposure to Ag:Ab complexes (Ravetch and Kinet, 1991; Van de Winkel and Capel, 1993; Fanger et al., 1996; Ravetch, 1997; Tew et al., 1997). There is also evidence suggesting that the affinity of FcγRI for IgG_{2a} and IgG₃ is much greater than for IgG₁ or IgG_{2b} (Van de Winkel and Capel, 1993). These affinity differences might explain the ability of MAb 1G6 (IgG_{2a}) to enhance humoral immunity while MAbs PB136, 3D9 and 2C₈ (IgG₁) do not.

We also investigated whether co-administration of a plasmid encoding murine GM-CSF with pSLPSLRSV-LTR.SgD would augment humoral responses in the face of pre-existing titers of passive polyclonal antibody. Somewhat surprisingly, inclusion of plasmid GM-CSF appeared to inhibit, rather than enhance, the humoral immune response (Fig. 5.1 F) since GM-CSF has been demonstrated to perpetuate antigen uptake and processing capacity of epidermal Langerhans cells (Heufler et al., 1987; Morrissey et al., 1987; Sallusto and Lanzavecchia, 1994). This capacity has been suggested to play a direct role in the observed augmentation of seroconversion efficacy and both cell-mediated and humoral immunity in mice immunized with plasmid encoded antigen and co-administered GM-CSF (Xiang and Ertl, 1995; Conry et al., 1996a; Geissler et al., 1997; Kim et al., 1997; Lewis et al., 1997). We previously demonstrated that plasmid encoded GM-CSF augments seroconversion efficacy and the magnitude of serum antibody levels significantly when co-administered with suboptimal, and to a lesser extent, optimal doses of pSLRSV.SgD (see sections 4.3.2 and 4.3.4). The failure of GM-CSF to augment B-cell immunity in this case suggests that maintenance of peripheral dendritic cell antigen uptake and processing capacity does not play a significant role in the ability of these

animals to mount potent, rapid B-cell immunity despite the presence of passive titers.

Other issues to consider when using DNA-based vaccines in neonates include: separation of intrinsic adjuvanticity of the plasmid DNA from immunological recognition of B-cell epitopes, naivete of the neonatal immune system and safety. Considerable evidence has arisen recently regarding the potential contribution of hypomethylated CpG motifs, adjacent sequences and distant poly G sequences in the recognition, uptake and adjuvanticity of bacterially derived DNA (Pisetsky, 1996a). Although the mechanism for recognition of immunostimulatory sequences (ISS's) has not been determined, it has been shown that absence or presence of these ISS's can result in reduction or enhancement of immune responses, respectively (Sato et al., 1996; Roman et al., 1997). It has also been demonstrated recently that these ISS's are responsible for induction of cytokines (IFN α , β , IL-12, IL-18) responsible for directing the ensuing immune response towards a Th1 type of response (Roman et al., 1997). There is currently little evidence regarding the longevity of the innate responses to ISS's, however, we have observed substantial elevations in IL-4 and IFN γ in draining lymph nodes, bone marrow and spleen 7 days following injection of a Null plasmid (unpublished data). Regardless, the adjuvant effects of hypomethylated plasmid DNA may have waned at the time that antigen-specific BCR on naive B-cells are able to "see" plasmid encoded antigen in the absence of coligation of Fc γ RIIB1. This problem is potentially compounded in species where the neonatal immunological naivete requires low doses of vaccine to avoid inducing a tolerance or profound immune deviation. It is possible that this problem of temporal separation of adjuvancy from antigen recognition by B-cells can be eliminated

by co-administering plasmid encoded immunodeviating cytokines such as IL-18 or IL-12, however, considerable work must be done before advocating use of plasmid encoded cytokines in neonates. It may be possible that ballistic delivery may provide an mechanism whereby the issue of segregation of plasmid adjuvancy effects from productive B-cell recognition of antigen is avoided. However, the method of DNA vaccine delivery must be considered in the context of specific pathogens and the character of the normal protective immune response (Mosmann and Sad, 1996; Zinkernagel, 1996). We must not ignore the benefits of Th1-like T-cell responses and CTL activity and characteristic ability of DNA vaccines to elicit either or both of these types of immunity. It has been demonstrated that potent CTL responses against measles virus haemagglutinin and fusion proteins can occur despite a profound suppression of B-cell responses by pre-existing passive titers (Galletti et al., 1995). Ultimately, T-cell responses may provide an avenue of effective immunization that is more achievable than the elicitation of B-cell responses in the face of suppressive maternal titers. These responses would, at the very least, decrease the time of disease susceptibility by providing a primed population of helper T-cells at the time of boosting.

There are a variety of experimental approaches that may increase the immune responses in the presence of passive antibody titers to an even greater levels than described in this article. Many of these involve the creation of molecular chimeras comprised of antigen and moieties that either prevent signalling through the B-cell FcγRIIB1 or provide additional positive signalling that could, hypothetically, override negative signals delivered through co-ligation of the BCR and FcγRIIB1 receptors.

Prevention of signalling through co-ligation of BCR and Fc γ RIIB1 is best described by fusions of complement fixing antibodies such as IgM or IgG_{2a} in mice or direct fusion of complement component C3d or iC3b to the antigen in question (Dempsey et al., 1996). Although the specific receptor for IgM has not been identified it has been demonstrated that engagement of this antibody by antigen can enhance humoral responses in the face of passive titers of IgG (Reitan and Hannestad, 1995). We, of course, have demonstrated in this article the potential for the complement fixing IgG_{2a} to circumvent the suppressive effects of passive IgG, however, the specific mechanism remains unknown. Induction of a positive signal that may override negative signalling might also be accomplished by the creation of a molecular fusion of CD40 ligand (CD154) to antigen or inclusion of this sequence within the same plasmid encoding the antigen. CD40 engagement has been demonstrated to be the critical, necessary and sufficient co-stimulatory signal to drive naive B-cells to secrete IgM and initiate maturation towards isotype switched plasma cells or memory B-cells (Gray et al., 1994; Han et al., 1995).

In summary, our data suggests that mice immunized once with a plasmid encoding a secreted form of BHV-1 gD were able to mount both humoral and cell-mediated immune responses in the face of pre-existing passive antibodies. However, the apparent enhancement of humoral responses, following immunization with a suboptimal dose of a conventional vaccine, suggests that a factor (or factors) within the passive sera administered to recipient mice may be responsible for the observed humoral immunity in this model. It is also possible that the strain of mouse, adjuvant VSA3 and/or dose of recombinant truncated gD can play pivotal roles in the enhanced seroconversion

described for C57BL/6 mice. Despite this problem these data provide direction for future experimentation addressing the potential of DNA-based vaccines in the presence of passive antibody.

6.0 GENERAL DISCUSSION AND CONCLUSION

When I began this project, no single publication describing immune responses to DNA-based vaccines existed in scientific literature. There were several published accounts describing uptake and expression of naked plasmid DNA encoding reporter genes, by myocytes following i.m. injection into muscle tissue (Wolff et al., 1990; Wolff et al., 1991). There was also some exciting preliminary data, initiated at VIDO, that demonstrated the potential for delivery of plasmid encoded antigens to elicit significant levels of immunity in mice and cattle. Since the commencement of this project, well over 400 hundred scientific articles have been published, in refereed journals, describing aspects of this most recent immunological phenomenon.

In this thesis I extended the initial work by Dr. Graham Cox, who demonstrated that immunization of mice and cattle with plasmids encoding bovine herpesvirus (BHV)-1 glycoproteins B, C and D resulted in significant levels of humoral immunity and indications that cell-mediated immunity was also elicited (Cox et al., 1993). I also assessed the ability of plasmids encoding eight different murine cytokines (IL-1 α , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN γ , TNF α) to augment or modulate immune responses to co-administered plasmids encoding a secreted form of BHV-1 gD. In addition, immune responses were characterized in passively immune mice following immunization with

plasmids encoding a secreted form of BHV-1.

The first objective of this thesis was to create several efficient expression plasmids encoding membrane anchored, secreted or cytosolic versions of BHV-1 gD, and establish that these constructs generated proteins that occurred in the appropriate cell compartments. The full length cDNA for BHV-1 gD (membrane anchored) had previously been cloned, sequenced and placed in an expression cassette (Tikoo et al., 1990). A secreted version of gD had also been described previously (Tikoo et al., 1993). A cytosolic version of BHV-1 gD was generated by deleting the signal peptide from the cDNA encoding the secreted version of gD, and all three cDNAs were subcloned, with gene regulatory elements, into a high copy number plasmid backbone. This allowed maximal yield, following amplification in DH-5 α bacterial hosts, and eliminated concerns over plasmid batch differences within single experiments. Following transient transfection of COS-7 cells with each the plasmids we established that each version of gD occurred primarily within the cell compartment for which the cDNAs had been designed (Fig. 3.3).

Creation of membrane anchored, cytosolic and secreted forms of the same protein allowed the investigation of immune responses to plasmid encoded antigens and allowed assessment of how the cell compartment in which antigens occurred could affect the subsequent immune character following i.m. immunization. Female C3H/HeN mice all developed substantial levels of serum antibodies and splenic cytokine profiles showing a prevalence of IFN γ (Fig. 3.4, 3.5, 3.6 and 3.10). Although IFN γ plays a significant role in the isotype switch to IgG_{2a} only mice immunized with cell-associated versions of gD

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(cytosolic and membrane anchored) displayed a predominance of serum IgG₂ (Figs. 3.7, 3.8, 3.9). Mice immunized with plasmids encoding the secreted form of gD displayed a predominance of serum IgG₁ which appeared to be a result of the influence of higher levels of IL-4 production in the draining lymph node of immune mice (Figs. 3.7, 3.8, 3.9 and 3.13). Thus it appeared that the cell compartment in which the antigen primarily occurred was able to modulate both humoral and cell-mediated immune responses.

In an effort to extend studies on immune modulation, plasmids encoding the secreted version of gD were co-administered with plasmids encoding a variety of murine cytokines previously shown to augment or deviate immune responses. Conventional molecular techniques were utilized to subclone cDNAs for IL-2 and GM-CSF into the same plasmid driving expression of the secreted version of gD (Material and Methods, 4.2.4). RT-PCR, in conjunction with site specific mutagenesis, of the total RNA or mRNA preparations from a variety of cell sources was utilized to generate IL-1 α , IL-4, IL-6, IL-10, IFN γ and TNF α , cDNAs which were subcloned into the same plasmid utilized to express the secreted version of gD (Materials and Methods, 4.2.4). Following restriction analysis, partial dideoxy sequencing and functional bioassay confirmation of cytokine identity, a preliminary experiment was carried out using a suboptimal dose of plasmids encoding the secreted version of BHV-1 gD co-administered with an equal dose of each of the plasmids encoding the cytokines (Fig. 4.3). Data from this experiment demonstrated that co-administration of plasmids encoding GM-CSF enhanced mean serum titers and the seroconversion efficacy of immunized mice (Table 4.4). Plasmids encoding murine TNF α , IL-4 and IL-6 also enhanced the magnitude of serum IgG

levels but with unique kinetics (Fig. 4.3 and Table 4.4). Co-administration of plasmids encoding IFN γ and TNF α increased the levels of serum IgG_{2a} in some, but not all, immunized mice (Fig. 4.4 and Table 4.5). In a follow-up experiment an optimal, fixed, dose of plasmid expressing BHV-1 SgD was co-administered with a two different doses of plasmids encoding GM-CSF or IFN γ (Fig. 4.6). Moderate doses of GM-CSF reproducibly enhanced early mean titers. High doses of GM-CSF resulted in a suppression of humoral responses, however, boosting with high doses of this cytokine resulted in an extremely potent memory response resulting in very high serum titers and a surprising increase in the levels of serum IgG_{2a} (Fig. 4.7 and Table 4.7). Assessment of cytokine secretion, within draining lymph nodes and the spleen, showed that co-administration of plasmids encoding GM-CSF induced significant increases in IL-4 secretion after boosting (Tables 4.8 A and B). Co-administration of plasmids encoding IFN γ resulted in the reduction of antibody responses, at moderate doses, that persisted regardless of boosting (Fig. 4.6). Conversely, mice immunized with high doses of this cytokine showed enhanced mean serum antibody levels and benefited from boosting (Fig. 4.6). Mice immunized with both moderate and high doses of plasmids encoding IFN γ displayed elevations in mean serum IgG_{2a} (Fig. 4.7 and Table 4.7).

The final objective of this thesis was to investigate the potential for DNA vaccines to overcome suppression of active humoral immunity (specifically B-cell mediated immunity) caused by preexisting passive titers of antigen-specific antibody (Muta et al., 1994). Certain unique features of DNA-based vaccines such as longevity of expression (Wolff et al., 1992) and the ability to transfect peripheral, and possibly secondary

lymphoid resident, antigen presenting cells (Raz et al., 1994; Condon et al., 1996) suggested that this vaccination approach may be more effective than conventional methods of immunization. Immunization of 7-8 week old female C3H/HeN or C57BL/6 mice, in the face of varying levels of passive titers of anti-gD antibodies resulted in seroconversion of mice within 2-4 weeks of immunization (Figs. 5.1 D, 5.3 C and D, and 5.5 C). Subsequent experiments employing monoclonal antibodies of the IgG₁ or IgG_{2a} isotype suggested that the rapid and profound development of active antibody titers in the presence of pre-existing high titers of passive polyclonal antisera may have occurred because of IgG_{2a} levels within the polyclonal antisera (Fig. 5.3 E,F and G). Literature indirectly supports this observation with evidence that passive titers of IgM can enhance the development of active antibody responses and that complement fixation is a necessary feature of this humoral immunity (Henry and Jerne, 1968; Heyman et al., 1988; Reitan and Hannestad, 1995).

The data provided in this thesis, and uncovered during the course of these studies, has provided important information on the impact of antigen form, co-administered plasmid encoded cytokines, and passive antibodies on the immune responses following immunization with plasmids encoding various forms of BHV-1 gD. Data provided and presented during the course of this work has, as often occurs with scientific investigations, raised many more questions and laid the groundwork for future investigations. Ongoing, and future directions, for studies include:

(i) Obviously, the importance of plasmid based vaccines in mouse models is significant

as an early indicator of the potential for success and information regarding the immune character, however, the full utility of this vaccine technology can only be realized by testing in the appropriate target species. For example, serum antibody levels are poor in cattle following i.m. delivery to the gluteal muscle mass, however, i.d. delivery to the skin of the ear greatly enhances this response (Cox et al., 1993; van Drunen Littel-van den Hurk et al., 1998). Furthermore, as observed in the mouse, the cellular compartment in the which the antigen occurs can have a profound impact on the immunological outcome following immunization of cattle.

(ii) Although the data presented in this thesis discusses immune responses following i.m. immunization with DNA-based vaccines, literature and studies carried out by Drs. Ralph Braun and Sylvia van Drunen Littel-van den Hurk have demonstrated the advantages of i.d. delivery of plasmids encoding antigens by injection and ballistic protocols (Braun et al., 1997; van Drunen Littel-van den Hurk et al., 1998). These data have led many scientists to investigate the mechanisms of immunity to DNA vaccines and the specific, and critical, cell types involved in presentation of plasmid encoded antigens to naive T- and B-cells within secondary lymphoid tissues . Preliminary evidence regarding the contribution of myocytes transfected with plasmids encoding antigens following i.m. immunization is controversial. While the question of direct involvement in immune priming by transfected myocytes has been resolved, questions revolving around the contribution of these antigen depots to the post prime development and maintenance of immune responses is very much at issue (Ulmer et al., 1996c; Feltquate et al., 1997). It

has been inferred in this thesis that injection site transfection of cells is of critical immunological importance and that direct transfection of cells within draining lymph nodes immediately following introduction of DNA occurs ineffectively and contributes little to immunity (Figs. 3.1.1, 3.1.2 and 3.1.3). Scientists continue to address the question of immune mechanism following delivery of DNA-based vaccines by each of the several methods available to introduce naked DNA vaccines into animals.

(iii) Preliminary work presented in this thesis contributed, in part, to the establishment of research projects at VIDO that address the mechanism by which GM-CSF and IL-4 augment immune responses to co-delivered antigen. The impact of these two cytokines, delivered i.d. in a sheep model, on the afferent lymphatic cell population character and dynamics, as well as, draining lymph node histological architecture and immunocytochemical profile are currently being assessed. Similarly, some initial work uncovered during the course of my studies, suggested the possibility that a surprising level of intrinsic adjuvanticity was associated with Null plasmids utilized in this thesis (data not presented in this thesis). The subsequent appearance of articles in refereed journals describing immune stimulatory sequences comprised of hypomethylated CpG motifs within bacterially derived deoxyribonucleic acids, laid the groundwork for ongoing studies, at VIDO, of these sequences in a sheep model (Krieg et al., 1995; Pissetsky, 1996a; Klinman et al., 1997; Roman et al., 1997).

(iv) Data presented in this thesis suggested that extracellular forms of antigens enhanced

the early kinetics and magnitude of humoral immunity to BHV-1 gD (Table 3.2). Subsequent studies in cattle supported this work to some extent and indicated that cattle responded more efficiently to plasmids encoding a secreted form of BHV-1 gD following i.d. immunization (van Drunen Littel-van den Hurk et al., 1998). This information was utilized to set the direction for the development of a DNA-based vaccine encoding a protective antigen (VP7) from the bovine rotavirus. VP7 normally encodes a protein with an endoplasmic localizing signal sequence that prevents secretion of this protein until it has associated with several other components (VP4 and VP6) of the viral capsid (Shaw et al., 1996). Creation of plasma membrane anchored and secreted forms of VP7, by Dr. S. Suradhat, have resulted in enhanced expression of these proteins as demonstrated by *in vitro* transfection of COS 7 cells. Despite the absence of detectable immune responses to these novel constructs, experience with gD, cytokine augmentation of immunity, and literature, have allowed the identification a variety of logical approaches to solve the problem of antigenicity from this unique viral protein.

(v) It is well documented that young animals from immune mothers are protected, in part, from infectious diseases by the passive transfer of maternal antibodies through colostrum and the placenta (species dependent)(MacDonald, 1992; Xiang and Ertl, 1992). We felt that some unique features of DNA-based vaccines justified a preliminary investigation into the potential to circumvent the suppressive effects of passively acquired antibodies on development of active immunity following immunization. Extension of data presented in this thesis is currently being assessed in mouse models and target

species. Future direction should include: assessment of immunity following immunization at different times following acquisition of passive titers, studies regarding the potential for chimeric antigens to avoid co-ligation of B-cell receptors and FcγRIIB1 receptors (IgM:Ag chimers) or redirection of antigen towards non B-cell Fc receptors, such as the high affinity FcγRI receptor on dendritic cells (anti-CD64 Fab:Ag fusions). It may also be practical to ignore attempts to elicit B-cell responses in the face of passive titers and to focus on cell-mediated immunity in these young animals. Certainly, DNA-based vaccines possess a unique capacity to drive potent CD4+ and CD8+ T-cell responses following immunization of animals.

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